VACCINATION:

TIPPING THE BALANCE
FROM PATHOGEN TO HOST

Mark Ariaans

Printing of this thesis was financially supported by:

Intervet B.V.

BD Biosciences

ISBN: 978-90-393-4889-5

Printed by Druk & Vorm, Nijmegen

Cover-design: V. Quigley

VACCINATION:

TIPPING THE BALANCE
FROM PATHOGEN TO HOST

Vaccinatie:

de balans kantelen
van pathogeen naar gastheer

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof**,**
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 23 september 2008
des middags te 4.15 uur

door

Markus Petrus Ariaans

geboren op 5 mei 1977
te Nijmegen

Promotor:	Prof. dr. W. van Eden
Co-promotoren:	Dr. E.J. Hensen Dr. L. Vervelde

Table of contents

Abbreviations

^{51}Cr	Chromium-51
APC	antigen presenting cell / allophycocyanin
ARD	avian respiratory disease
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate / p nitro blue tetrazolium chloride
BSA	bovine serum albumin
cfu	colony-forming units
ChIFN-γ	chicken interferon-gamma
CMI	cell-mediated immunity
ConA	Concanavalin A
DC	dendritic cell
dpi	days post inoculation (with *Escherichia coli*)
EID_{50}	median embryo infective dose
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunosorbent Spot assay
FBS	fetal bovine serum
FI	fluorescence intensity
FIPV	Feline Infectious Peritonitis Virus
FITC	fluorescein-isothiocyanate
IBV	Infectious Bronchitis Virus
ICCS	intracellular cytokine staining
IFN-γ	interferon-gamma
i.t.	intratracheal
MHC	Major Histocompatibility Complex
MHV	Mouse Hepatitis Virus
NDV	Newcastle Disease Virus
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
pfu	plaque-forming units
PMA	Phorbol 12-Myristate 13-Acetate
PRR	pattern recognition receptor
qPCR	quantitative Real-Time PCR
RPE	R-phycoerythrin
SFV	Semliki Forest Virus
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramentylbenzidine

CHAPTER 1

General introduction

1. General outline
2. Innate immune defences
3. Adaptive immunity
4. Respiratory infections
5. Strategy
6. Scope of this thesis

1. General outline

Vaccination has been a very successful strategy in dealing with a wide range of infectious diseases for many decades. This success has led to a broader use for vaccines as a tool for immune manipulation for other diseases like autoimmune diseases and cancer. But in this thesis we will restrict ourselves to the original aim of protection against infectious diseases.

The desired effect of vaccination is to produce memory immune cells from naïve precursor cells (Campos and Godson, 2003), in order to provide long-lasting, protective immunity against a specific micro-organism. This is achieved by exposing a subject to either live attenuated or killed micro-organisms, but also to subunit vaccines such as purified toxoids, or alternative ways of antigen presentation to the immune system (Leclerc, 2003). Although often very successful, these vaccination approaches have also failed on numerous occasions, for reasons that are generally not known. Some vaccines will provide short-term protection but no memory; other vaccines give rise to good immune memory but fail to elicit a protective response upon challenge with the pathogen. A good or strong immune response is not a guarantee for protective immunity. For example, respiratory infections are notoriously problematic in inducing protective immunity (Ghedin et al., 2005; Power, 2008; Van Boven et al., 2000). From a vaccine development perspective, infections that generate a solid immune response without the induction of protective immunity are an interesting research area.

In the field of immunology a wide range of novel research tools has become available recently, which led to new insights in host-pathogen interactions and a greater understanding of the molecular basis of protective immunity. An adequate immune response is not only the result of antigen specificity, but the nature of the response is also largely influenced by the context during its induction. Therefore, the focus of vaccine development can be shifted from antigen specificity alone to antigen specificity plus context. New knowledge and technology may allow us to tailor vaccines to combat infectious diseases in a strategic way and most suited for optimal protection and long-lasting immune memory.

Vaccinology is not restricted to a particular species, as is the case in many other fields of medicine. It is a field where strategies can be shared optimally between humans and animal species. Studies in livestock and companion animals have additional value compared to common experimental animals, because they are both the experimental and the target species at the same time. We have focussed on the chicken to study effects of vaccine composition on the immunological response.

The chicken has traditionally been a popular research subject for studying immunological mechanisms (Cooper et al., 1965; Davison, 2008; Glick et al., 1956). With its well-defined humoral immune system and limited complexity of the Major Histocompatibility (MHC) complex, the monitoring of cell mediated immunity is facilitated (Kaufman et al., 1999). Therefore the chicken is an adequate choice to examine the parameters involved in ‘appropriate’ modulation of immune responses by vaccines. Its specific immune responses are very similar to the responses in mammalian species, so extrapolation is possible. In addition, the chicken is an economically highly relevant species and a great demand exists for vaccines that provide adequate protection against avian pathogens. Important poultry vaccine targets are the respiratory pathogens, which are also problematic target pathogens in humans and our main area of interest, as mentioned previously. Therefore the

chicken has the great benefit of being an experimental animal and a target animal for the vaccine at the same time (Kaufman, 2000).

2. Innate immune defences

Innate immunity has for a long time been considered as merely a first line of immune defence, displaying anti-microbial and pro-inflammatory activity that was mostly or even entirely unrelated to the type of adaptive immune response. Its main role was thought to be keeping the pathogen at bay until the components of the adaptive immune system were alerted and recruited. This view has drastically changed over the past years. It is now generally accepted that the innate immune system has a key role in regulating the sensitivity and specificity of the adaptive immune response (Guy, 2007; Pulendran and Ahmed, 2006). The innate immune system is in fact capable of recognising certain molecular patterns that are associated with specific pathogen groups via so-called pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD proteins. Dendritic cells (DCs) play a critical role in processing information from these receptors and subsequently modulating the adaptive immune response.

The innate immune system of birds probably has different characteristics than that of mammals, and in this light is an interesting research target for observing alternative ways in which to approach the host defence against respiratory infections between species. In contrast to mammals, a healthy avian respiratory system has very few macrophages residing in the lumen of the respiratory system (Qureshi, 2003; Toth et al., 1987). Upon infection, heterophils and macrophages rapidly enter the respiratory tract to engulf the infectious agent, though this influx appears to be largely absent after stimulation with non-immunogenic substances (Toth, 2000). It has been reported that epithelial cells in the parabronchi have phagocytic ability, which might help to clear such inert contaminating particles from the respiratory tract (Stearns, 1987). The heterophils and macrophages display a range of non-specific defensive reactions to bacteria and fungi, such as phagocytosis, oxidative burst, and production of nitric oxide (NO). Their response to viral infections is less straight-forward; some viruses impair macrophage responses, others actually enhance them. DCs respond to viruses by producing large amounts of type-I interferons and process and present viral antigens to cells of the adaptive immune system (Toth, 2000).

Although many aspects of macrophage and DC responses to viral infection are still poorly understood, the outcome of these responses has an effect on disease progression and therefore an important impact on vaccination strategies and risk assessment studies.

3. Adaptive immunity

Knowledge about the interactions of the adaptive immune responses in mammalian species is extensive. With regards to vaccination, it is important to emphasize that long-lasting protective immunity depends entirely on the development of antigen-specific immune memory after first encounter with the infectious agent. The context in which the specific immune cells are triggered has a crucial influence on the outcome of a specific response. Therefore, correct antigen specificity is a prerequisite for success, but not necessarily sufficient. If we intend to study variations in the “context” of immunisation we should have an excellent control

on the antigen-specific determinants involved. Keeping the complexity of the antigen specificity as low as possible will allow for other components, such as adjuvants, to be introduced during a study to examine their effect on the resulting immune response. If the antigen-specific components are too complex, it will be very difficult to distinguish between antigen-specific effects on the immune response and effects resulting from the immunisation context.

Adaptive immune responses in the chicken appear to be comparable to the mammalian system, suggesting the possibility of extrapolating techniques from mammalian research to avian research, and vice versa. An additional benefit in studying antigen-specific immune responses in the chicken is its well-characterised MHC complex, called the “B complex” in the chicken (Kaufman et al., 1999). Differences in MHC (or “B”) molecules intrinsically change the specificity of the T cell receptor. Therefore, studies on variations in the context of immunisation ideally should be performed in animals with identical MHC types to start with.

As in mammals, adaptive immune responses in birds can be skewed towards a cell-mediated response, involving $CD4^+$ T_h1 cells and $CD8^+$ cytotoxic T cells, or a humoral response, involving $CD4^+$ T_h2 cells and B cells. These responses are characterized by different sets of cytokines and innate immune cells, and the nature of the adaptive immune response is highly dependent on the type of pathogen and on the innate immune signals that arise from the initial encounter with this pathogen (Degen et al., 2005; Vandaveer et al., 2001).

The humoral immune response provides good protection against respiratory pathogens, by blocking or otherwise interfering with the initial infection. Pathogens that evade the humoral response and infect cells of the respiratory tract are targeted by antigen-specific effector T cells, which lyse the infected cells and produce inflammatory cytokines, predominantly interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) (Cavanagh, 2007; Hikono et al., 2006). Besides these relatively short-lived effector cells, a pool of memory B and T cells is also generated. The memory B and T cells persist in the animal long after their target pathogen has been cleared and are capable of rapid generation of effector functions after re-exposure to the pathogen (Ahmed and Gray, 1996; Pulendran and Ahmed, 2006). These cells form the basis for long-lasting protective immunity.

4. Respiratory infections

Before presenting the vaccination strategies we have in mind, it is appropriate to go into more detail about the players we intend to use in our studies.

In the case of respiratory infections, surviving the natural infection does not always give rise to long-lasting protective immunity (Braciale, 2005). In addition these respiratory infections, and in particular virus infections, are notoriously problematic to prevent by using vaccination. There are several reasons for this. One important aspect is the ability of many respiratory pathogens to evade humoral immune responses that interfere with the initial infection. Influenza viruses do this for example by rapidly altering their surface antigens, making them undetectable to antibodies raised against earlier variants of these viruses (Carrat and Flahault, 2007; Escorcia et al., 2008). Vaccines that consist of inactivated or live attenuated versions of the virus would therefore quickly be rendered ineffective.

It is obvious from the above that an effective and long-lasting immune response to these pathogens requires cell-mediated immunity as well as humoral immunity. Cell-mediated immunity (CMI), although not able to block the initial

infection, does reduce its severity (Belshe et al., 2000; Flynn et al., 1998). It can also target more conserved pathogenic proteins that are not accessible to antibodies, which allows for the generation of vaccines against a wider range of virus strains and with a lower chance of becoming obsolete due to viral antigen shift.

It has been observed that CMI responses to some pathogens in the respiratory tract wane in time (Aguas et al., 2006; Liang et al., 1994; Paunio et al., 2000) for largely unknown reasons. It could be that this limited protection lifespan is somehow linked to the immunosuppressive environment in the respiratory tract, which is important because damage to the respiratory epithelium could provide entry routes for pathogens (Borron et al., 1998; Toth, 2000). Furthermore, it has been shown that there are several subsets of memory T cells, and we do not know at present which of these subsets need to be generated in order to obtain long-lasting protective immunity.

4.1. Poultry

Respiratory infections in poultry have a serious economic effect on the poultry industry worldwide. Avian respiratory diseases (ARD) are mainly caused by mycoplasma, viruses, and bacteria. A large range of mycoplasma including *M. gallisepticum* and *M. synoviae* affect commercial poultry (Noormohammadi, 2007). Most viral ARDs are induced by Infectious Bronchitis Virus (IBV), Newcastle Disease Virus (NDV) , Avian Influenza Virus (AIV) , Infectious Laryngotracheitis Virus (ILV), and pneumoviruses (Villegas, 1998). Important bacterial pathogens include *Pasteurella multocida* (fowl cholera), *Bordetella avium* (bordetellosis), and *Escherichia coli* (colibacillosis) (Glisson, 1998). Although each of these infectious agents can cause an ARD on its own, the most serious diseases arise from combined infections of two or more of these pathogens (Toth, 2000).

In both mammals and birds, vaccination strategies for respiratory infections are occasionally failing. This means that vaccination studies in chickens are attractive from a research perspective, by giving insights into immune responses after vaccination or infection, but also from an economic perspective, as there is a great demand for vaccines against a wide range of poultry diseases.

The avian respiratory system not only regulates gas exchange between the body and the environment, but it is also involved in flight. As a result, there are several key differences between the lung morphology of birds (parabronchial) and mammals (bronchoalveolar), and consequently between the defence mechanisms required for adequate protection against respiratory pathogens.

In contrast to the mammalian lungs, where freshly inhaled air is mixed with residual air in the airways, the avian lung is a flow-through system. The nasal cavity is the first line of defence against microorganisms. It contains an expanded, mucous-covered epithelial surface with cilia that quickly transport any particles that are trapped in the mucous to the pharynx for disposal. Likewise, the trachea, primary bronchi and the upper part of the secondary bronchi are lined with ciliated epithelium to transport trapped particles to the nasal cavity (Fedde, 1998).

Upon inhalation, air travels via the larynx through the trachea, which splits into two primary bronchi. Each of these bronchi enter a lung and traverse the whole lung to the caudal air sacs. Secondary bronchi branch off from the primary bronchus towards other air sacs, and in turn have many tertiary bronchi (parabronchi) branching off. Located mainly at the junction of the primary bronchus and caudal secondary bronchi and in the areas where the parabronchi open up into the air sacs are the Bronchus-associated lymphoid tissues (BALTs), which are organised

lymphoid structures (Reese et al., 2006). They contain a large number of lymphocytes, macrophages, heterophils, and DC-like cells. The majority of them are B cells and $CD4^+$ αβ T cells, forming the parafollicular caps, with some $CD8^+$ cells scattered throughout the tissue (Jeurissen et al., 1994). BALT formation in poultry takes place over a period of several weeks, but already at five days after hatching diffuse infiltration of leukocytes can be found surrounding the parabronchi (Jeurissen et al., 1989). In the interstitial lung tissue, the majority of lymphocytes are B cells and $CD8^+$ αβ T cells, although some $CD4^+$ and γδ T cells are also present.

The avian lungs are much more rigid structures than mammalian lungs. The relatively high rigidity of the avian lung allows for a large increase in respiratory surface area, without the risk of collapse of the airway capillaries (Maina, 2002; Reese et al., 2006). This provides efficient gas exchange with the blood in a very small space. On the downside, this large surface area of the lung, coupled with its very thin blood-gas barrier, also provides a convenient entry route for pathogens and can be damaged easily by environmental particles.

The lung-associated air sacs operate as bellows; by changing the volume of the air sacs, pressure differences are created across the lungs which cause the air to move through the system. The air sacs take up most of the area inside the body cavities not occupied by other organs. Their volume can be changed by contraction and subsequent elastic recoil of the muscles in the body wall (Fedde, 1998). This setup allows large volumes of air to be moved efficiently through the avian respiratory system in one direction, as opposed to the mammalian system in which inhaled air resides in the lungs for a relatively short time before being exhaled through the same route. However, it also means that inhaled particles are not easily expelled again.

When studying respiratory immune responses in poultry it is important to keep in mind such differences between the avian and the mammalian airways, for they have important implications for the demands placed on the immune defences and on vaccine development.

4.2. Pathogens of interest

4.2.1. Infectious Bronchitis Virus

Infectious Bronchitis (IB) is an acute, highly contagious respiratory disease in poultry that is caused by the avian coronavirus Infectious Bronchitis Virus. The disease is characterised by tracheal rales, coughing and sneezing, often accompanied by nasal discharge. Respiratory symptoms of IBV infection usually last for less than seven days. In addition, some IBV strains affect the reproductive and renal systems (Cavanagh, 2007).

IB has a significant economic impact. It can cause poor weight gain and condemnation at processing in broilers, and in laying birds it may result in decreased egg production and quality. Respiratory and renal symptoms can result in mortality in young chicks. Although most IBV strains are not lethal, mortality can arise due to secondary bacterial infections, primarily in broilers (Ignjatovic and Sapats, 2000; Matthijs et al., 2003).

Upon histological examination of an IBV-infected bird, lesions are characteristically found in the trachea. Virus replication takes place in ciliated epithelial cells lining the bronchi, which results in deciliation and detachment of epithelial cells, oedema, hyperplasia, and infiltration of the submucosa by mononuclear cells (Ignjatovic and Sapats, 2000).

IBV is a member of the family *Coronaviridae*, which also includes viruses such as Severe Acute Respiratory Syndrome (SARS) virus (Cavanagh, 2003), Feline Infectious Peritonitis Virus (FIPV) (de Groot-Mijnes et al., 2005), and Murine Hepatitis Virus (MHV) (Ae et al., 2002). It is a single-stranded RNA virus, consisting of four structural proteins: a spike (S) protein and a membrane (M) protein, which together with the smaller envelope (E) protein form the viral membrane, and a nucleocapsid (N) protein which is associated with the viral RNA (Collisson et al., 2000; Sapats et al., 1996). The N protein plays an important role in virus assembly and replication.

A wide range of IBV strains have been isolated from all over the world. Whereas these strains vary greatly in their S protein structure, the N protein is highly conserved between strains. Its limited structural complexity and relation to SARS, combined with its economical impact on the poultry industry both directly as a disease agent and due to its predisposing effects on bacterial superinfections such as colibacillosis, make IBV an interesting and relevant research target.

4.2.2. Escherichia coli

Often an IBV infection is followed by a secondary bacterial infection with avian pathogenic *Escherichia coli* (APEC), causing prolonged and enhanced respiratory symptoms which can last for several weeks (Dho-Moulin and Fairbrother, 1999; Matthijs et al., 2003). The immunological mechanisms underlying this phenomenon will be an important area of study in this thesis.

Colibacillosis is one of the main causes of economic losses in the poultry industry worldwide. It affects hatching and egg production, and can cause mortality (septicaemia) and condemnation at processing. Infection is thought to occur mainly by inhalation of faeces-contaminated dust. Following infection, the bacteria adhere to respiratory epithelial cells and then enter the bloodstream through the lower respiratory tract (the lungs and the air sacs) (Pourbakhsh et al., 1997). Secondary infection with *E. coli* can lead to inflammation of the posterior air sacs (airsacculitis), the pericardium (pericarditis), and the peritoneum and tissues around the liver (perihepatitis) (Vandekerchove et al., 2004).

5. Strategy

The aim of this thesis is to find strategies to develop vaccines for protection against pathogens that do not induce adequate protective immunity after natural infection or after vaccination with the infectious agent. To reach this objective, three main areas needed to be controlled:

- detailed monitoring of antigen specific responses by both B cells and T cells, and the resulting antibodies,
- measuring qualitative and quantitative variations in the induction method by using antigens of reduced complexity. Only when the specificity is under full control, comparisons can be made,
- stimuli to modulate the nature of a response independent of specificity.

These three points will be addressed here in relation to the target species of our choice, the chicken.

5.1. Monitoring

Currently immunological tools to detect and quantify T cell responses in the chicken are limited, so our first aim is to develop T cell assays. This information will

allow us to study the specific immune responses after presentation of specifically selected pathogen components.

Although various tools are available to measure innate immune responses in the chicken, methods to analyse the adaptive immune responses in poultry have to be extended to fit our goals. A steadily growing number of avian cytokines is being identified and cloned, making it possible to use molecular techniques such as quantitative real-time PCR to study the adaptive immune response (Lowenthal et al., 2000; Wigley and Kaiser, 2003). The availability of reliable bio-assays and ELISAs in poultry research is unfortunately more limited. Well-established methods to measure T cell responses in mammals, based on intracellular cytokine staining or enzyme-linked immunosorbent Spot (ELISPOT) analysis, have not yet been extensively developed for the chicken. Our first task was therefore to develop these assays to measure IFN-γ production, which is a widely used marker for T cell activity (Lambrecht et al., 2000). These immunological methods will allow us to determine the effect of factors such as selected pathogen components and route of administration on the adaptive immune response.

5.2. Antigens of low complexity

The second aim is to study the context of the immune response using antigens of limited complexity. To study the influence of the context of immunisation, the number of antigenic determinants shared by the “experimental vaccine” and the infectious agent should be as small as possible. Otherwise it would become impossible to interpret the results in relation to specificity. Direct administration of purified pathogen components is often unsuccessful, due to incorrect processing or lack of immunostimulatory factors. Therefore, not only the selected component but also the way in which it is presented to the immune system is vital in obtaining the desired immune response.

Our original plans were to use selected peptides containing one or few involved epitopes of the infectious agent. This however turned out to be problematic due to the limited immunogenicity of the peptides, which meant that no discernable immune recall response could be found. An alternative that could solve the problem of lack of immunogenicity might be obtained using DNA technology. The gene containing the epitopes of interest can be incorporated in a pathogen-unrelated immunogenic carrier plasmid and used for DNA immunisation.

Apart from the improved immunogenicity, DNA immunisation has an additional advantage. Many currently available poultry vaccines elicit a good humoral response, but no adequate cell-mediated response. However, several economically important poultry diseases such as Avian Leukosis Virus (ALV; Thacker et al., 1995), Reticulo-endotheliosis Virus (REV; Weinstock et al., 1989) and IBV (Seo and Collisson, 1998) do require cytotoxic T cell responses to clear virus and induce protection. DNA-based vaccines have an advantage over conventional vaccines in that they induce both humoral and cell-mediated immunity (Hassett et al., 2000), without the risk of reversal to virulence that inactivated or live attenuated vaccines may pose. Also, DNA-based vaccines have been shown to work in newborn chicks, whereas traditional vaccines often fail due to a poorly developed immune system and undesired interaction with maternal antibodies (Kapczynski et al., 2003).

In designing a vaccination strategy against IBV, the nucleocapsid protein is an interesting target for vaccine development because, unlike the membrane-associated spike (S) protein, it is highly conserved between different IBV strains. As

successful vaccination against IBV requires both humoral and cell-mediated immune responses, a DNA plasmid expressing the IBV nucleocapsid protein seems a good vaccine candidate to obtain long-lasting protective immunity against a range of IBV strains.

5.3. Modulation of responses

The third aim of this thesis is to get a better understanding of how the immune system can be modified to respond to the chosen vaccine components and vaccination route in a way that leads to the best protective effect. It has been widely observed that not only different adjuvants, but also other respiratory pathogens that happen to be present in the animal around the time of vaccination, can completely change the course of the immune response. This can lead to totally different outcomes even when the vaccine used is the same (Didierlaurent et al., 2007).

A well documented and economically highly relevant example of undesired modulation of the avian immune response by a pathogen is the predisposition for severe colibacillosis by experimental infection or vaccination with IBV (Matthijs et al., 2005; Nakamura et al., 1992; Vandekerchove et al., 2004). We will use this infection model to study the parameters involved in the enhanced susceptibility to colibacillosis, because it is a good example of immunomodulation by the agent that we have used as target pathogen for our other studies and an established infection model (Matthijs et al., 2003) with potentially major benefits to the poultry industry.

6. Scope of this thesis

As mentioned previously, the aim of this thesis is to extend our knowledge of various parameters involved in vaccine design. In the previous section we have identified the three topics that in our view are instrumental to the development of a successful vaccine. Each of these topics will be studied in more detail in the following chapters.

In chapter 2, results are presented of the development of assays that allow for more detailed monitoring of the avian immune response. The techniques that we will focus our attention on are the ELISPOT and intracellular cytokine staining (ICCS) assays for the analysis of T cell responses.

When testing these immunological tools for their functionality in infection studies, we made an unexpected observation with regard to IBV. It appeared that IBV induced rapid activation of chicken immune cells, regardless of previous exposure of the birds to this pathogen. This observation was investigated further, in order to determine whether the IFN-γ production was due to an artefact of our newly developed tools or an actual biological phenomenon. These studies are the subject of chapter 3.

In chapter 4, a DNA vaccine strategy is described that is based on limited complexity of the antigenic determinants in combination with the aim to target both the humoral and the cell-mediated immune response.

In chapter 5 and 6, studies to gain insight into the mechanisms behind modulation of the immune response are described that are based on a well known clinical finding. We investigated the mechanisms underlying the enhanced susceptibility to colibacillosis of broilers after infection or vaccination with IBV.

Finally, the findings described in this thesis and their relevance to vaccine development and the understanding of the immunological mechanisms involved are discussed in chapter 7.

References

Ae, M., Sr, W., Paterson, Y., 2002, Murine hepatitis virus--A model for virus-induced CNS demyelination. J. Neurovirol. 8, 76-85.
Aguas, R., Gonçalves, G., Gomes, M.G., 2006, Pertussis: increasing disease as a consequence of reducing transmission. Lancet Infect. Dis. 6, 112-117.
Ahmed, R., Gray, D., 1996, Immunological Memory and Protective Immunity: Understanding Their Relation. Science 272, 54-60.
Belshe, R., Gruber, W., Mendelman, P., Mehta, H., Mahmood, K., Reisinger, K., Treanor, J., Zangwill, K., Hayden, F., Bernstein, D., Kotloff, K., King, J., Piedra, P., Block, S., Yan, L., Wolff, M., 2000, Correlates of Immune Protection Induced by Live, Attenuated, Cold-Adapted, Trivalent, Intranasal Influenza Virus Vaccine. J. Infect. Dis. 181, 1133-1137.
Borron, P.J., Crouch, E.C., Lewis, J.F., Wright, J.R., Possmayer, F., Fraher, L.J., 1998, Recombinant Rat Surfactant-Associated Protein D Inhibits Human T Lymphocyte Proliferation and IL-2 Production. J. Immunol. 161, 4599-4603.
Braciale, T.J., 2005, Respiratory Syncytial Virus and T Cells: Interplay between the Virus and the Host Adaptive Immune System. Proc Am Thorac Soc 2, 141-146.
Campos, M., Godson, D.L., 2003, The effectiveness and limitations of immune memory: understanding protective immune responses. Int. J. Parasitol. 33, 655-661.
Carrat, F., Flahault, A., 2007, Influenza vaccine: The challenge of antigenic drift. Vaccine 25, 6852-6862.
Cavanagh, D., 2003, Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol. 32, 567-582.
Cavanagh, D., 2007, Coronavirus avian infectious bronchitis virus. Vet. Res. 38, 281-297.
Collisson, E.W., Pei, J., Dzielawa, J., Seo, S.H., 2000, Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev. Comp. Immunol. 24, 187-200.
Cooper, M.D., Peterson, R.D., Good, R.A., 1965, Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken. Nature 205, 143-146.
Davison, F., 2008, The importance of the avian immune system and its unique features, In: Davison, F., Kaspers, B., Schat, K.A. (Eds.) Avian Immunol. Academic Press, pp. 1-12.
de Groot-Mijnes, J.D.F., van Dun, J.M., van der Most, R.G., de Groot, R.J., 2005, Natural History of a Recurrent Feline Coronavirus Infection and the Role of Cellular Immunity in Survival and Disease. J. Virol. 79, 1036-1044.
Degen, W.G.J., Daal, N.v., Rothwell, L., Kaiser, P., Schijns, V.E.J.C., 2005, Th1/Th2 polarization by viral and helminth infection in birds. Vet. Microbiol. 105, 163-167.
Dho-Moulin, M., Fairbrother, J.M., 1999, Avian pathogenic Escherichia coli (APEC). Vet. Res. 30, 299-316.
Didierlaurent, A., Goulding, J., Hussell, T., 2007, The impact of successive infections on the lung microenvironment. Immunology 122, 457-465.
Escorcia, M., Vazquez, L., Mendez, S., Rodriguez-Ropon, A., Lucio, E., Nava, G., 2008, Avian influenza: genetic evolution under vaccination pressure. Virol. J. 5, 15.
Fedde, M.R., 1998, Relationship of structure and function of the avian respiratory system to disease susceptibility. Poult. Sci. 77, 1130-1138.
Flynn, K.J., Belz, G.T., Altman, J.D., Ahmed, R., Woodland, D.L., Doherty, P.C., 1998, Virus-Specific CD8+ T Cells in Primary and Secondary Influenza Pneumonia.

Immunity 8, 683-691.
Ghedin, E., Sengamalay, N.A., Shumway, M., Zaborsky, J., Feldblyum, T., Subbu, V., Spiro, D.J., Sitz, J., Koo, H., Bolotov, P., Dernovoy, D., Tatusova, T., Bao, Y., St George, K., Taylor, J., Lipman, D.J., Fraser, C.M., Taubenberger, J.K., Salzberg, S.L., 2005, Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. Nature 437, 1162-1166.
Glick, B., Chang, T.S., Jaap, R.G., 1956, The bursa of Fabricius and antibody production. Poult. Sci. 35, 2.
Glisson, J.R., 1998, Bacterial respiratory disease of poultry. Poult Sci 77, 1139-1142.
Guy, B., 2007, The perfect mix: recent progress in adjuvant research. Nat. Rev. Micro. 5, 505-517.
Hassett, D.E., Slifka, M.K., Zhang, J., Whitton, J.L., 2000, Direct ex vivo kinetic and phenotypic analyses of CD8(+) T-cell responses induced by DNA immunization. J. Virol. 74, 8286-8291.
Hikono, H., Kohlmeier, J.E., Ely, K.H., Scott, I., Roberts, A.D., Blackman, M.A., Woodland, D.L., 2006, T-cell memory and recall responses to respiratory virus infections. Immunol. Rev. 211, 119-132.
Ignjatovic, J., Sapats, S., 2000, Avian infectious bronchitis virus. Rev. Sci. Tech. 19, 493-508.
Jeurissen, S.H., Vervelde, L., Janse, E.M., 1994, Structure and function of lymphoid tissues of the chicken. Poult. Sci. Rev., 183-207.
Jeurissen, S.H.M., Janse, E.M., Koch, G., Boer, G.F., 1989, Postnatal development of mucosa-associated lymphoid tissues in chickens. Cell Tissue Res. 258, 119-124.
Kapczynski, D.R., Hilt, D.A., Shapiro, D., Sellers, H.S., Jackwood, M.W., 2003, Protection of chickens from infectious bronchitis by in ovo and intramuscular vaccination with a DNA vaccine expressing the S1 glycoprotein. Avian Dis. 47, 272-285.
Kaufman, J., 2000, The simple chicken major histocompatibility complex: life and death in the face of pathogens and vaccines. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 355, 1077-1084.
Kaufman, J., Milne, S., Gobel, T.W., Walker, B.A., Jacob, J.P., Auffray, C., Zoorob, R., Beck, S., 1999, The chicken B locus is a minimal essential major histocompatibility complex. Nature 401, 923-925.
Lambrecht, B., Gonze, M., Meulemans, G., van den Berg, T.P., 2000, Production of antibodies against chicken interferon-gamma: demonstration of neutralizing activity and development of a quantitative ELISA. Vet. Immunol. Immunopathol. 74, 137-144.
Leclerc, C., 2003, New approaches in vaccine development. Comp. Immunol. Microbiol. Infect. Dis. 26, 329-341.
Liang, S., Mozdzanowska, K., Palladino, G., Gerhard, W., 1994, Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. J. Immunol. 152, 1653-1661.
Lowenthal, J.W., Lambrecht, B., van den Berg, T.P., Andrew, M.E., Strom, A.D., Bean, A.G., 2000, Avian cytokines - the natural approach to therapeutics. Dev. Comp. Immunol. 24, 355-365.
Maina, J.N., 2002, Structure, function and evolution of the gas exchangers: comparative perspectives. J. Anat. 201, 281-304.
Matthijs, M.G., van Eck, J.H., de Wit, J.J., Bouma, A., Stegeman, J.A., 2005, Effect of IBV-H120 vaccination in broilers on colibacillosis susceptibility after infection with

a virulent Massachusetts-type IBV strain. Avian Dis. 49, 540-545.
Matthijs, M.G., van Eck, J.H., Landman, W.J., Stegeman, J.A., 2003, Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. Avian Pathol. 32, 473-481.
Nakamura, K., Cook, J.K., Frazier, J.A., Narita, M., 1992, Escherichia coli multiplication and lesions in the respiratory tract of chickens inoculated with infectious bronchitis virus and/or E. coli. Avian Dis. 36, 881-890.
Noormohammadi, A.H., 2007, Role of phenotypic diversity in pathogenesis of avian mycoplasmosis. Avian Pathol. 36, 439-444.
Paunio, M., Hedman, K., Davidkin, I., Valle, M., Heinonen, O.P., Leinikki, P., Salmi, A., Peltola, H., 2000, Secondary measles vaccine failures identified by measurement of IgG avidity: high occurrence among teenagers vaccinated at a young age. Epidemiol. Infect. 124, 263-271.
Pourbakhsh, S.A., Boulianne, M., Martineau-Doize, B., Fairbrother, J.M., 1997, Virulence mechanisms of avian fimbriated Escherichia coli in experimentally inoculated chickens. Vet. Microbiol. 58, 195-213.
Power, U.F., 2008, Respiratory syncytial virus (RSV) vaccines--Two steps back for one leap forward. J. Clin. Virol. 41, 38-44.
Pulendran, B., Ahmed, R., 2006, Translating innate immunity into immunological memory: implications for vaccine development. Cell 124, 849-863.
Qureshi, M.A., 2003, Avian macrophage and immune response: an overview. Poult. Sci. 82, 691-698.
Reese, S., Dalamani, G., Kaspers, B., 2006, The avian lung-associated immune system: a review. Vet. Res. 37, 311-324.
Sapats, S.I., Ashton, F., Wright, P.J., Ignjatovic, J., 1996, Novel variation in the N protein of avian infectious bronchitis virus. Virology 226, 412-417.
Seo, S.H., Collisson, E.W., 1998, Cytotoxic T lymphocyte responses to infectious bronchitis virus infection. Adv. Exp. Med. Biol. 440, 455-460.
Stearns, R., Barnas, GM, Walski, M, Brain JD, 1987, Deposition and phagocytosis of inhaled particles in the gas exchange region of the duck, Anas platyrhynchos. Resp. Physiol. 67, 23-36.
Thacker, E.L., Fulton, J.E., Hunt, H.D., 1995, In vitro analysis of a primary, major histocompatibility complex (MHC)-restricted, cytotoxic T-lymphocyte response to avian leukosis virus (ALV), using target cells expressing MHC class I cDNA inserted into a recombinant ALV vector. J. Virol. 69, 6439-6444.
Toth, T.E., 2000, Nonspecific cellular defense of the avian respiratory system: a review. Dev. Comp. Immunol. 24, 121-139.
Toth, T.E., Siegel, P., Veit, H., 1987, Cellular defense of the avian respiratory system. Influx of phagocytes: elicitation versus activation. Avian Dis. 31, 861-867.
Van Boven, M., de Melker, H.E., Schellekens, J.F.P., Kretzschmar, M., 2000, Waning immunity and sub-clinical infection in an epidemic model: implications for pertussis in The Netherlands. Math. Biosci. 164, 161-182.
Vandaveer, S.S., Erf, G.F., Durdik, J.M., 2001, Avian T helper one/two immune response balance can be shifted toward inflammation by antigen delivery to scavenger receptors. Poult. Sci. 80, 172-181.
Vandekerchove, D., Herdt, P.D., Laevens, H., Butaye, P., Meulemans, G., Pasmans, F., 2004, Significance of interactions between Escherichia coli and respiratory pathogens in layer hen flocks suffering from colibacillosis-associated mortality. Avian Pathol. 33, 298-302.

Villegas, P., 1998, Viral diseases of the respiratory system. Poult Sci 77, 1143-1145.
Weinstock, D., Schat, K.A., Calnek, B.W., 1989, Cytotoxic T lymphocytes in reticuloendotheliosis virus-infected chickens. Eur. J. Immunol. 19, 267-272.
Wigley, P., Kaiser, P., 2003, Avian cytokines in health and disease. Rev. Bras. Cienc. Avic. 5, 1-14.

CHAPTER 2

ELISPOT and intracellular cytokine staining: novel assays for quantifying T cell responses in the chicken

Mark P. Ariaans[1], Peter M. van de Haar[1], John W. Lowenthal[2], Willem van Eden[1], Evert J. Hensen[1], Lonneke Vervelde[1]

[1]Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[2]CSIRO, Livestock Industries, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria, 3220 Australia

Developmental and Comparative Immunology, 2008; 32: 1398-1404

Abstract

The measurement of T cell responses in chickens, not only for quantitative aspects but also for the qualitative nature of the responses, becomes increasingly important. However, there are very few assays available to measure T cell function. Therefore, we have developed ELISPOT and an intracellular cytokine staining (ICCS) assay. ELISPOT assay for the detection of ChIFN-γ production was set up and shown to be reproducible for both polyclonal and antigen-specific stimuli such as Newcastle disease virus (NDV). However, the ELISPOT assay lacks the ability to identify individual cytokine producing cells. Separation of $CD4^+$ and $CD8^+$ T cell populations gave additional information, but appeared to have the disadvantage of a loss of cell interactions during stimulation. In a further refinement, individual cells were identifiable by ICCS, which gives the possibility to characterize for multiple characteristics, such as cytokine production and phenotype of the cell. Using ICCS, ChIFN-γ production was evaluated. Although cells were detected at only low frequencies, polyclonal stimulation of PBMC or spleen cells resulted in a significant increase in ChIFN-γ production by $CD4^+$ and $CD8^+$ cells.

Key words: T cell assay, ELISPOT, interferon-gamma, chicken

1. Introduction

Qualitative and quantitative methods to study T-cell responses in mammals, such as intracellular staining of cytokines and Enzyme-Linked Immunosorbent Spot (ELISPOT), have not yet been properly established in avian research. These techniques provide highly sensitive and quantitative analysis of T cell activity and above all the ability to study the nature of T cell responses at the single-cell level. Intracellular staining of cytokines has the added advantage that qualitative analysis can be performed through labelling of cell surface markers, allowing identification of the specific cell subpopulations that contribute to cytokine production. By depletion of specific subsets of T cells using cell sorting methods additional information on the responding cells might also be found with the ELISPOT assay.

The purpose of this study was to develop an ELISPOT assay and intracellular cytokine staining (ICCS) for the detection of chicken interferon-gamma (ChIFN-γ) as novel tools to examine avian T cell responses, and to test their functionality in the study of host-pathogen interactions.

IFN-γ plays an important role in cell-mediated immune responses. ChIFN-γ production is used as an indicator for cell-mediated immune activity in various avian disease models [1, 2]. In this study, birds immunized with a commercial Newcastle Disease Virus (NDV) vaccine were used to test whether anti-viral responses could be measured. A role for cell-mediated immunity (CMI) has been shown in NDV infections, where proliferation of (mainly $CD8^+$) T cells was observed in response to NDV vaccination [3, 4].

2. Materials and methods

2.1. Animals

Adult commercial White Leghorn and Silver Nick chickens were housed in groups and fed ad libitum on commercial feed. Chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

2.2. Virus

The NDV used for *in vitro* recall activation was obtained as commercial freeze-dried vials which contained at least 10^6 EID_{50} per dose (Nobilis; ND clone 30; batch: 031046D, Intervet, The Netherlands).

The chickens were divided in two groups, a vaccine group and a control group. At five weeks of age, chickens were vaccinated with Nobilis Newcavac (Intervet) i.m. and live ND clone 30 by eye-drop, according to recommendation. The spleens were isolated at 17 days post vaccination. All inocula were prepared in LPS-free PBS prior to use at a concentration of 10^6 EID_{50}/ml.

2.3. Preparation of splenocytes

Spleen tissue was squeezed through 70 µm mesh in RPMI1640 culture medium containing 2% FCS to prepare a single cell suspension. Splenocytes were isolated by density gradient centrifugation for 20 min at 850 x g using FICOLL-Hypaque 1.078, washed twice with phosphate-buffered saline (PBS, Cambrex) and adjusted to 3 x 10^6 cells/ml in culture medium (RPMI1640 medium supplemented with 10% FCS, 2 mM glutamax-I, 50 mM β-mercaptoethanol (β-ME) and 100 U/ml

penicillin/streptomycin).

2.4. ELISPOT assay

MAIPS4510 MultiScreen^{TM}-IP 96-well plates (Millipore, Billerica, USA) were coated with 5 µg/ml mouse-anti-ChIFN-γ (CAC1233; Biosource International, California, USA) in coating buffer (sodium bicarbonate, 50 mM, pH 9.6) for 2 h at 37°C, 5% CO_2, or overnight at 4°C. All incubation steps were performed with 100 µl/well. Plates were washed twice with blocking buffer (RPMI 1640 supplemented with 2% FCS, 2 mM glutamax-I, 100 U/ml P/S, 50 µM β-ME) and incubated with blocking buffer for 1 h at 37°C, 5% CO_2. The blocking buffer was discarded and splenocytes were seeded at 3 x 10^5 cells/well in triplicate in culture medium. Cells were incubated in the presence of either culture medium or medium supplemented with one of the following stimuli to a final volume of 200 µl per well: Phorbol 12-myristate 13-acetate (PMA) and ionomycin, Concanavalin A (ConA; Sigma), or NDV (ND clone 30). The cells were incubated for 24 or 48 h at 41°C, 5% CO_2. Subsequently the plates were washed twice with distilled water, and three times with washing fluid (PBS supplemented with 0.1% Tween-20). ChIFN-γ was detected by incubation with 1 µg/ml biotinylated mouse-anti-ChIFN-γ (CAC1233; Biosource International) in assay buffer (PBS supplemented with 0.1% Tween-20 and 1% BSA; 100 µl/well) for 1 h at room temperature. Plates were washed four times with washing fluid and incubated with 2 µg/ml streptavidin-alkaline phosphatase (Sigma; 100 µl/well) in assay buffer for 1 h at room temperature. Plates were washed five times with washing fluid and the assay was developed using BCIP/NBT substrate (Roche, Basel, Switzerland). The plates were then washed with copious amounts of tap water, air dried and analyzed using the A·EL·VIS machine and the Eli.Analyse software (Version 4.0) that allows for automated counting of the number of spots based on size and intensity.

2.5. Magnetic bead depletion

Splenocytes were labeled with mouse anti-chicken CD4 (CT-4) or CD8α (CT-8; Southern Biotechnology Associates, Inc., Birmingham, USA) in MACS buffer (PBS + 1% FCS + 2 mM EDTA) for 20 min on ice. Cells were washed twice with MACS buffer, resuspended with goat anti-mouse IgG MicroBeads (Miltenyi Biotec, GmbH) and cell depletion was performed using a magnetic separation column according to the manufacturer's instructions. The cells were counted and seeded in duplicate in ELISPOT wells coated with mAb anti-ChIFN-γ (as described previously) at 3 x 10^5 cells/well. Cells were incubated for 48 h at 41°C, 5% CO_2 in culture medium, or medium supplemented with ConA, or PMA and ionomycin. ChIFN-γ-producing cells were detected using the ELISPOT protocol as described above. Samples of each depleted cell population were taken to analyse bead depletion efficiency using flow cytometry.

2.6. ELISA for ChIFN-g detection

A total of 2 x 10^6 splenocytes per chicken were incubated for 48 h in a 24-well plate in 500 µl of culture medium at 41°C, 5% CO_2, without stimulation or in the presence of ConA. Afterwards, supernatants were tested for ChIFN-γ using a commercial sandwich ELISA kit (Biosource International, California, USA) according to manufacturer's instructions.

2.7. Intracellular staining of ChIFN-γ

Splenocytes were seeded in duplicate in 96-well round bottomed plates (Corning Costar, NY, USA; 3799) at 10^6 cells/well in culture medium, and stimuli in culture medium were added to a final volume of 200 µl per well. The cells were incubated at 41°C, 5% CO_2. After 2 h incubation Brefeldin A (Sigma; 5 µg/ml) was added and incubation continued for another 2 h. The cells were washed and labeled with fluorescein (FITC)-labeled or phycoerythrin (PE)-labeled mouse anti-chicken CD8α (CT-8), CD8β (EP42) or CD4 (CT-4; Southern Biotechnology Associates). Cells were washed twice with FACS buffer and incubated with Perm/Fix solution (Becton Dickinson, Franklin Lakes, USA) for 20 minutes on ice. Cells were washed by incubation with Perm/Wash solution (Becton Dickinson), supplemented with 0.25% normal mouse serum (NMS). Cells were intracellularly stained with mouse-IgG anti-ChIFN-γ antibody mAb80 [5] labeled with allophycocyanin (APC). Cells were washed with Perm/Wash solution with 0.25% normal mouse serum (NMS) followed by a wash step with FACS buffer, and the cell pellets were resuspended in FACS buffer. Cells were acquired on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) using Cell Quest software. Data analysis was done with FlowJo software (Treestar, Ashland, OR).

2.8. Statistical analysis

Statistical analysis was performed using SPSS version 12.0.1 for Windows. Analysis of data was performed using a paired T-test or one-way ANOVA. Results were considered statistically significant if $p \leq 0.05$.

3. Results

3.1. ChIFN-γ ELISPOT using mitogen stimulation

To set up the ELISPOT assay mitogen stimulated splenocytes were used. ChIFN-γ production was measured at 48 h after stimulation with either ConA or PMA and ionomycin (figure 1A). A highly significant ($p < 0.0001$) increase in the number of spots was found after mitogen stimulation when compared to unstimulated splenocytes.

To test the specificity of the spots, we compared ELISPOT plates coated with antibodies against either chicken IFN-γ or bovine IFN-γ. Chicken splenocytes and bovine PBMCs were incubated for 24 h in culture medium with or without ConA or PMA and ionomycin. Production of IFN-γ was only detected with IFN-γ-specific antibodies of the same species-specificity as the coating antibodies (figure 1B). High spot counts were found in wells with chicken splenocytes and anti-ChIFN-γ or bovine PBMC with anti-bovine IFN-γ antibodies (a gift from dr. A. Koets). Wells with chicken splenocytes and anti-bovine IFN-γ antibodies were negative, confirming the absence of non-specific interactions between the culture supernatant and the well surface.

To test whether the ELISPOT results coincide with an existing assay to quantify ChIFN-γ at protein level, ChIFN-γ production was measured using both ELISPOT and ELISA (figure 2). Though it must be taken into account that the correlation between number of cells that produce ChIFN-γ in the ELISPOT assay and the quantity of soluble ChIFN-γ as measured by ELISA is unknown, in both assays all birds showed a significant increase in ChIFN-γ after ConA stimulation.

To evaluate the contribution of different cell subpopulations to the ChIFN-γ response, $CD4^+$ and/or $CD8\alpha^+$ splenocytes were depleted, ensuring depletion of both CD8αα and CD8αβ subpopulations, before performing the ELISPOT assay.

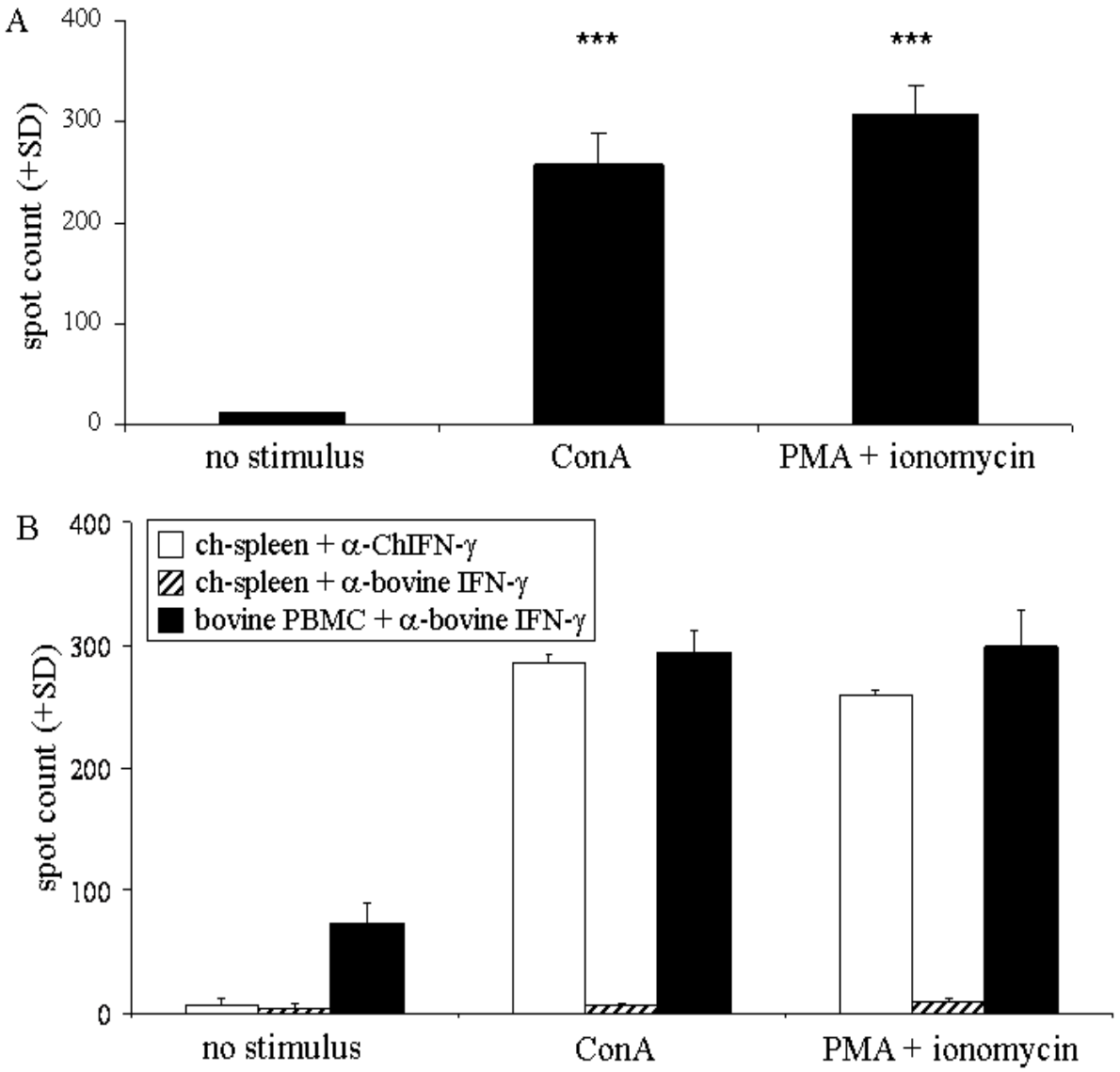

Figure 1. *ELISPOT analysis of stimulated T cells. (A) Splenocytes were added in triplicate to ChIFN-γ-coated wells and stimulated for 48 h with medium, ConA (10 µg/ml) or PMA (100 ng/ml) and ionomycin (500 ng/ml). Spot counts represent the number of ChIFN-γ secreting cells. *** Significantly different from unstimulated group ($p < 0.0001$; n=9). (B) Chicken splenocytes were stimulated for 24 h with ConA (10 µg/ml), or PMA (100 ng/ml) and ionomycin (500 ng/ml), in wells coated with antibodies against chicken (white bars) or bovine (hatched bars) IFN-γ. Bovine PBMC were incubated under the same conditions for 24 h in wells coated with bovine IFN-γ antibodies (black bars).*

Depletion with magnetic beads was between 93 and 96% efficient (figure 3A).

After depletion of $CD4^+$ or $CD8\alpha^+$ T cells, ChIFN-γ production decreased considerably ($p < 0.001$ for both depletions) in splenocyte cultures stimulated with PMA and ionomycin (figure 3B). ChIFN-γ production was almost completely absent in ConA-stimulated splenocyte cultures after depletion ($p < 0.0001$).

3.2. ChIFN-γ production after vaccination

Splenocytes from NDV-vaccinated and unvaccinated chickens were stimulated with NDV for 24 h and tested for ChIFN-γ production using the ELISPOT

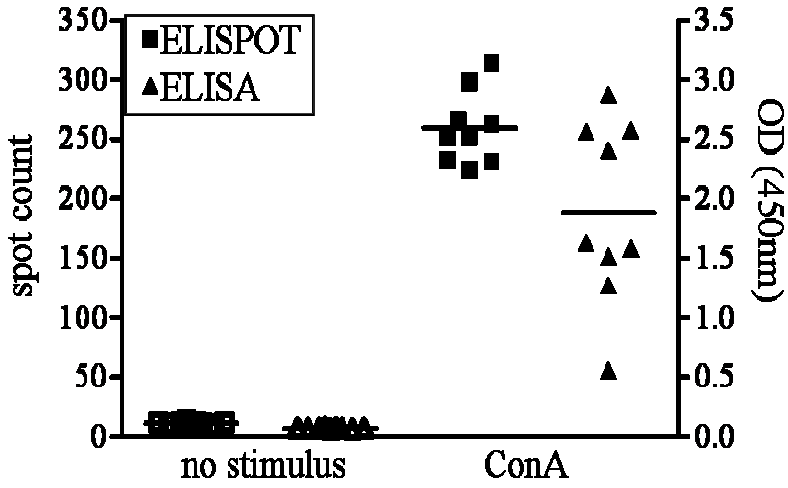

Figure 2. *ChIFN-γ production measured using ELISA and ELISPOT. Splenocyte cultures (n=9) were stimulated for 48 h in culture medium with or without ConA (10 µg/ml). ELISPOT results for each bird (squares) are depicted as spot counts (left y-axis). ELISA results for each bird (triangles) are depicted as OD readings at 450 nm (right y-axis).*

assay (figure 4). An increase in ChIFN-γ production was found after restimulation with NDV, and a significantly higher number of spots were found using splenocytes of NDV-vaccinated chickens compared to splenocytes of unvaccinated chickens ($p < 0.05$).

3.3. Intracellular staining of ChIFN-γ

Another powerful but demanding technique to study T cell responses is the intracellular staining of cytokines combined with surface staining of lymphocytes at single cell level. Splenocytes were stimulated for 4 h with PMA and ionomycin or ConA and double-stained for T cell markers and ChIFN-γ. A representative example is shown in figure 5A, B and C. The results show a significant ($p < 0.05$) increase in ChIFN-γ production by $CD4^+$ and $CD8\alpha^+$ cells compared to cells stained with the isotype control (figure 5D).

Based on the fluorescence intensity (FI) of CD8α, the $CD8\alpha^{int}$ positive cells were relatively more frequent ChIFN-γ^+ than the $CD8\alpha^{hi}$ positive cells (figure 5B plot 2). The cells were considered to be $CD8\alpha^{int}$ based on their FI ranging from approximately 13 to 60 and $CD8\alpha^{hi}$ with a FI of 60 to 290 (gates based on FI are shown in figure 5B and 5C plot 1). After triple staining for CD8α, CD8β and ChIFN-γ, the results indicated that both $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ cells produce ChIFN-γ (figure 5C plot 3). Based on the percentage cells that were $CD8\alpha^{int}$ or $CD8\alpha^{hi}$ and the percentage cells that were ChIFN-γ^+ $CD8\alpha^{int}$ and IFN-γ^+ $CD8\alpha^{hi}$, we concluded that also in the triple stained cells the $CD8^{int}$ positive cells produce ChIFN-γ relatively more frequently (figure 5C plot 1 vs plot 3) than the $CD8\alpha^{hi}$. A significant downregulation of CD4 was found after 4 h stimulation with ConA or PMA, whereas CD8α was only downregulated after 4 h stimulation with ConA (figure 5E).

4. Discussion

In this paper we present a functional ELISPOT assay and an intracellular cytokine staining (ICCS) technique to measure ChIFN-γ production for analysis of both CD4 and CD8 T cell responses in chickens. IFN-γ production is routinely used as a marker for T cell activity in a wide range of species. The ELISPOT and ICCS assays are established methods for measuring IFN-γ production in other species such as mice, humans, cats and horses [6-9], but are not yet available in poultry research. They have several advantages over other methods that are currently used

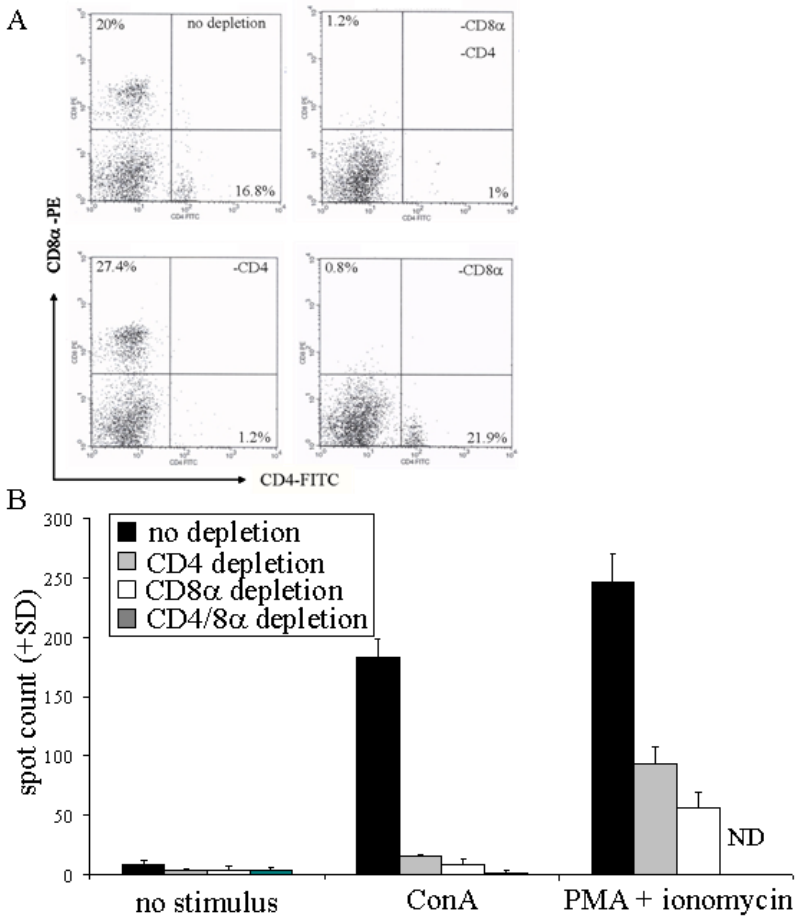

Figure 3. *(A) Representative illustration of depletion of chicken splenocyte subpopulations using magnetic beads. Depletion efficiency is shown for CD4 (bottom left), CD8α (bottom right), or both (top right) cell subpopulations, as compared to an undepleted splenocyte sample (top left).*
(B) Splenocytes (n=2) were incubated in triplicate for 48 h at 3 x 10^5 cells/well in ChIFN-γ-coated wells with medium, PMA (100 ng/ml) and ionomycin (500 ng/ml), or ConA (10 µg/ml). Spot counts represent ChIFN-γ-secreting cells from whole splenocyte cultures or depleted cultures as indicated. ND: not done.

to measure T cell responses in poultry. ELISPOT and ICCS are more discriminative and antigen-specific, and do not require radioactivity or lengthy procedures. Furthermore, the ICCS provides additional qualitative information that is not available using techniques as ELISA, qPCR, ^{51}Cr-release, and proliferation assays.

When combined with magnetic bead depletion, the ELISPOT assay could not provide information about the contribution of specific subpopulations to the immune response. This was possibly due to loss of antigen presenting cells during the depletion procedure. ICCS has the advantage that qualitative information can be obtained without the need for depletion of certain cell types, a treatment that can influence the immune response due to loss of cell interactions.

To exclude non-specific binding of ChIFN-γ to the ELISPOT plate membrane or the capture antibodies, we incubated chicken splenocytes in wells coated with

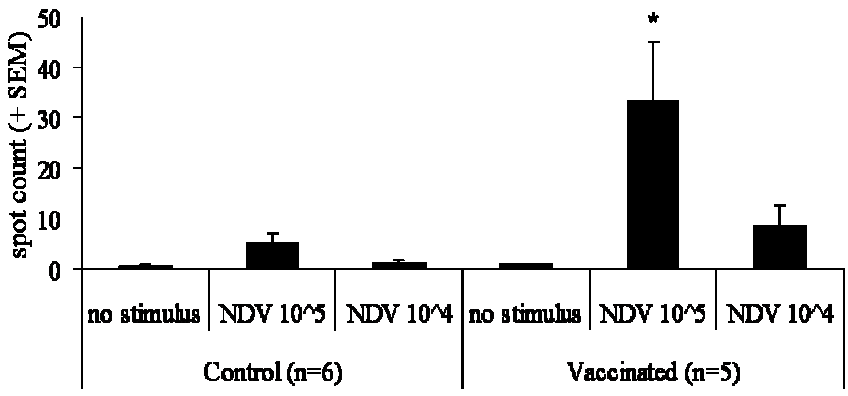

Figure 4. *Splenocytes were incubated in ChIFN-γ-coated wells for 24 h in medium with or without NDV (10^5 or 10^4 EID_{50}). Spots represent ChIFN-γ secreting cells. * Significantly different from control group ($p < 0.05$).*

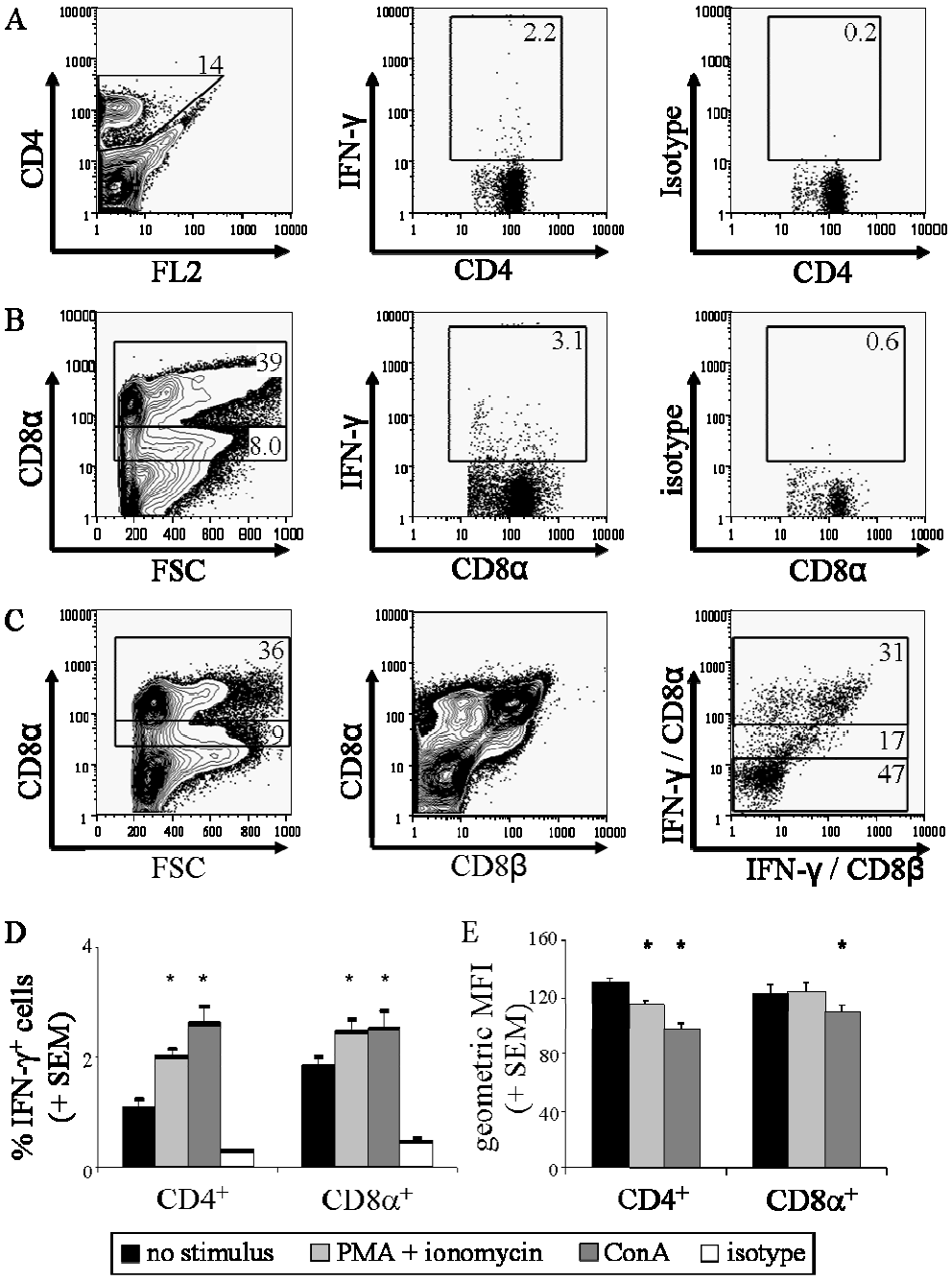

Figure 5. *Intracellular staining for ChIFN-γ of splenocytes. Chicken splenocytes were stimulated for 4 h with PMA (50 ng/ml) and ionomycin (500 ng/ml), and live cells were gated for CD4 (A) or CD8α expression (B). Representative staining of ChIFN-γ produced by CD4$^+$ cells (A plot 2) and CD8α$^+$ cells (B plot 2), compared to the isotype control (A and B plot 3). Cells were triple stained for CD8α, CD8β and ChIFN-γ (C). Definition of CD8α^{hi} and CD8α^{int} positive cells as indicated by the gating is shown in the first plot. Identification of CD8αα$^+$ and CD8αβ$^+$ cells is shown in the second plot. In the third plot, ChIFN-γ-producing cells expressing CD8β and/or CD8α are identified. The bottom left graph (D) shows the percentage of CD4$^+$ and CD8α$^+$ splenocytes that produce ChIFN-γ after stimulation with PMA and ionomycin, or with ConA (5 µg/ml) compared to an isotype control (+ SEM; n=11). Changes in geometric mean fluorescence intensity (MFI) of CD4$^+$ and CD8α$^+$ splenocytes after stimulation with PMA and ionomycin or ConA is shown in the bottom right graph (E; + SEM; n=11). Asterisks indicate statistical significance compared to isotype control (D) or unstimulated cells (E) ($p < 0.05$).*

antibodies directed against bovine IFN-γ. Spots were found in wells coated with anti-chicken, but not with anti-bovine IFN-γ mAbs, confirming that the spots were specific for ChIFN-γ-producing cells.

Although the ELISPOT technique provides us with a way to quantify the immune response, it does not give qualitative information about the type of immune cells involved. After depletion of cell subpopulations using magnetic beads, qualitative information could not be obtained in addition to quantitative information when using ELISPOT. A possible explanation for the higher number of spots found in depleted cultures after PMA and ionomycin stimulation than after ConA stimulation is that PMA and ionomycin stimulation drives a more generalized activation of immune cells (including $CD4^-$ and $CD8^-$ cells) without the need for costimulation [10], whereas ConA-induced activation may require help from other cell types such as APCs [11] that are potentially lost in the depletion procedure.

In the ICCS assay, where depletion of cell subpopulations is not required for qualitative analysis, $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ cells were shown to produce ChIFN-γ. The percentage $CD4^+$ and $CD8\alpha^+$ cells that produced ChIFN-γ increased significantly after stimulation with either ConA or PMA. Stimulation with ConA showed no difference between the percentage of ChIFN-γ-producing $CD4^+$ and $CD8\alpha^+$ cells when using ICCS. $CD8\alpha^{int}$ positive cells produced ChIFN-γ relatively more frequently than $CD8\alpha^{hi}$ cells. This could be due to downregulation of CD8α on the cell surface upon activation with ConA. However, after 4 h stimulation with PMA we did not observe a downregulation of CD8α on the cell surface. After stimulation with PMA, natural killer cells which are $CD8\alpha\alpha^+$ [12] might be responsible for the relatively higher frequency of IFN-γ^+ cells. However, we do not yet know whether these cells are $CD8\alpha^{int}$. Similar to human mononuclear cells, we saw downregulation of CD4 after 4 h stimulation with PMA and no downregulation of CD8α after 4 h stimulation with PMA [13], whereas both CD4 and CD8α were downregulated after 4 h stimulation with ConA.

To determine whether the ELISPOT assay is functionally applicable to the study of virus infections, we measured ChIFN-γ responses in splenocytes of chickens that had been previously vaccinated with Newcastle Disease Virus. After stimulation of splenocytes from NDV-vaccinated chickens with live NDV we measured a significant increase in the number of ChIFN-γ-producing cells, whereas no response was found in splenocyte cultures of unvaccinated chickens with the same dose of NDV.

We have developed convenient, sensitive and quantitative ELISPOT and ICCS assays for the chicken that can be used to measure ChIFN-γ responses induced by mitogens and additionally, in the case of the ELISPOT assay, for a selected pathogen. ICCS provides additional qualitative information on the cell subpopulations contributing to the response. These assays offer an alternative to assays such as ^{51}Cr-release, proliferation or qPCR assays in poultry. In the near future, we intend to adapt these assays for other cytokines as soon as antibodies become available.

Acknowledgements

We thank dr. Robbert G. van der Most and dr. Benedicte Lambrecht for their scientific comments and valuable discussion. Dr. L. Vervelde was financially supported by a BSIK VIRGO consortium grant (Grant no. 03012), The Netherlands.

References

[1] Kaiser P, Rothwell L, Galyov EE, Barrow PA, Burnside J, Wigley P, 2000. Differential cytokine expression in avian cells in response to invasion by Salmonella typhimurium, Salmonella enteritidis and Salmonella gallinarum. Microbiology 146, 3217-3226.
[2] Lambrecht B, Gonze M, Meulemans G, van den Berg TP, 2004. Assessment of the cell-mediated immune response in chickens by detection of chicken interferon-gamma in response to mitogen and recall Newcastle disease viral antigen stimulation. Avian Pathol. 33, 343-350.
[3] Russell PH, Dwivedi PN, Davison TF, 1997. The effects of cyclosporin A and cyclophosphamide on the populations of B and T cells and virus in the Harderian gland of chickens vaccinated with the Hitchner B1 strain of Newcastle disease virus. Vet. Immunol. Immunopathol. 60, 171-185.
[4] Seal BS, King DJ, Sellers HS, 2000. The avian response to Newcastle disease virus. Dev. Comp. Immunol. 24, 257-268.
[5] Lowenthal JW, Lambrecht B, van den Berg TP, Andrew ME, Strom AD, Bean AG, 2000. Avian cytokines - the natural approach to therapeutics. Dev. Comp. Immunol. 24, 355-365.
[6] Karlsson AC, Martin JN, Younger SR, Bredt BM, Epling L, Ronquillo R, et al, 2003. Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. J. Immunol. Methods 283, 141-153.
[7] Sirriyah J, Dean GA, LaVoy A, Burkhard MJ, 2004. Assessment of CD4+ and CD8+ IFN-gamma producing cells by ELISPOT in naive and FIV-infected cats. Vet. Immunol. Immunopathol. 102, 77-84.
[8] Kamath AT, Groat NL, Bean AG, Britton WJ, 2000. Protective effect of DNA immunization against mycobacterial infection is associated with the early emergence of interferon-gamma (IFN-gamma)-secreting lymphocytes. Clin. Exp. Immunol. 120, 476-482.
[9] Paillot R, Daly JM, Juillard V, Minke JM, Hannant D, Kydd JH, 2005. Equine interferon gamma synthesis in lymphocytes after in vivo infection and in vitro stimulation with EHV-1. Vaccine 23, 4541-4551.
[10] Van Seventer GA, Shimizu Y, Horgan KJ, Shaw S, 1990. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J. Immunol. 144, 4579-4586.
[11] Perrin PJ, Davis TA, Smoot DS, Abe R, June CH, Lee KP, 1997. Mitogenic stimulation of T cells reveals differing contributions for B7-1 (CD80) and B7-2 (CD86) costimulation. Immunology 90, 534-542.
[12] Göbel TW, Chen CL, Shrimpf J, Grossi CE, Bernot A, Bucy RP,
Auffray C, Cooper MD, 1994. Characterization of avian natural killer cells and their intracellular CD3 protein complex. Eur. J. Immunol. 24, 1685-1691.
[13] Kemp K, Bruunsgaard H, 2001. Identification of IFN-gamma-producing CD4+ T cells following PMA stimulation. J. Interf. Cytok. Res. 21, 503-506.

CHAPTER 3

Infectious Bronchitis Virus induces acute interferon-gamma production through polyclonal stimulation of chicken leukocytes

Mark P. Ariaans[1], Peter M. van de Haar[1], Evert J. Hensen[1], Lonneke Vervelde[1]

[1]Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

Manuscript submitted

Abstract

Infectious Bronchitis Virus, a member of the Coronaviridae, is a respiratory pathogen in poultry. We found that *in vitro* stimulation with IBV resulted in ChIFN-γ production in splenocytes of both infected birds and uninfected birds. The non-specific stimulation did not occur when other avian viruses or other coronaviruses were used or when mammalian cells were stimulated with IBV. Inactivation of IBV reduced ChIFN-γ production, but ChIFN-γ remained elevated compared to unstimulated cells. An increase in ChIFN-γ mRNA was detected in splenocytes from IBV-infected and uninfected chickens as early as one hour after stimulation with IBV. These results indicate that IBV acts as a polyclonal stimulus, inducing a rapid production of IFN-γ even without previous exposure to the virus. This rapid, possibly excessive, production of ChIFN-γ might be one of the causes of the massive influx of macrophages and $CD4^+$ T cells in the chicken lung after IBV infection.

Key words: Infectious Bronchitis Virus, ELISPOT, chicken, polyclonal stimulation, interferon-gamma, superantigen

1. Introduction

Infectious Bronchitis Virus is a coronavirus that causes an acute, highly contagious respiratory disease in poultry. IBV is a coronavirus of limited structural complexity which, as well as being an economically relevant pathogen in poultry, also bears close resemblance to the human pathogen Severe Acute Respiratory Syndrome coronavirus (SARS-CoV; Cavanagh, 2003). In order to study the immune responses to IBV in poultry in more detail, techniques are required that provide information about the immune cells participating to the response on a single-cell level. It has been shown that $CD8^+$ T cells play an important role in controlling IBV infection (Collisson et al., 2000). Furthermore, it is known that interferons are actively involved in the immune response against IBV (Hackney et al., 2003; Otsuki et al., 1988). Most of the techniques that are available to study T-cell responses in poultry do not provide any information about the response on single-cell level. We developed an ELISPOT assay and intracellular cytokine staining for the detection of ChIFN-γ production, which is a marker of T-cell activity (Ariaans et al., 2008b).

This ELISPOT assay allowed for the quantification of immune cell activity after mitogen stimulation and after vaccination with Newcastle Disease Virus (NDV), providing information about infection status of poultry and about the kinetics of the infection. An infection experiment was set up in which ChIFN-γ production by splenocytes from IBV-uninfected chickens was compared to that of IBV-infected chickens after *in vitro* restimulation with virulent IBV strain M41. Contrary to our expectations, the results indicated that ChIFN-γ production was not only significantly increased in splenocytes of IBV-infected chickens after *in vitro* restimulation with IBV, but also in splenocytes of IBV-uninfected chickens. Based on these findings, we investigated the possibility of polyclonal stimulation by IBV in more detail.

2. Materials and methods

2.1. Animals

Commercial White Leghorn chickens and Mycoplasma-free broiler chickens (Cobb) were housed in groups and fed ad libitum on commercial feed. Chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

2.2. Virus

The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, the Netherlands, as freeze-dried vials containing $10^{8.3}$ EID_{50}/1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. The IB vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials which contained at least $10^{3.0}$ EID_{50} (egg infective dose 50%) per dose (Nobilis® IB H120). Just prior to infection, all IBV inocula were prepared in distilled water and contained at least $10^{3.0}$ EID_{50}/ml of H120 virus and $10^{4.6}$ EID_{50}/ml of M41 virus. NDV was obtained as commercial freeze-dried vials which contained at least 10^6 EID_{50} per dose (Nobilis; ND clone 30). Feline Infectious Peritonitis Virus (FIPV) and Mouse Hepatitis Virus (MHV; both 10^8 pfu/ml) were a kind gift from the Virology Department of the Faculty of Veterinary Medicine, Utrecht University. Reticulo-Endotheliosis Virus (REV-C; 5 x 10^6 TCID/ml) was a kind gift from dr. G. Koch (CIDC-Lelystad, NL). All inocula were prepared in LPS-free PBS prior to *in vitro* stimulation at a concentration of 10^6 EID_{50}

or pfu/ml.
IBV M41 was purified using a sucrose gradient (Cornelissen et al., 1997; Koopmans et al., 1986). The gradient consisted of layers of 20-50% sucrose (top to bottom) in TES-V (20 mM TRIS-HCl at pH 7.3, 1 mM EDTA, 100 mM NaCl). This gradient was autoclaved, left to linearise at 4°C overnight, loaded with an IBV virus sample and centrifuged at 15,000 x g for 3 h at 4°C. Gradient fractions were collected from the bottom (50% sucrose solution) to the top (20%) with a fluid pump. For *in vitro* restimulation, inactivation of virus was achieved by direct exposure of the virus to UV illumination (~ 1 mW/cm^3) for 1 h (Ravindra et al., 2008; Stern and Sefton, 1982). Egg allantois fluid was a kind gift from dr. J. de Wit (GD-Animal Health Service, Deventer, NL).

2.3. ELISPOT assay

Preparation of splenocytes was described before in Ariaans et al. (2008b). Briefly, spleen tissue was squeezed through 70 µm mesh in RPMI1640 culture medium containing 2% FCS to prepare a single cell suspension. Splenocytes were isolated by density gradient centrifugation using FICOLL-Hypaque.
The ELISPOT assay has been described by Ariaans et al. (2008b). Briefly, MultiScreen^{TM}-IP 96-well plates were coated with mouse-anti-ChIFN-γ, blocked and splenocytes were seeded at 3 x 10^5 cells/well in triplicate in culture medium. Cells were incubated in the presence of either culture medium or medium supplemented with Concanavalin A (ConA; 10 µg/ml), phorbol 12-myristate 13-acetate (PA; 100 ng/ml) and ionomycin (500 ng/ml), or virus and incubated for 24 or 48 h at 41°C, 5% CO_2. ChIFN-γ was detected by incubation with biotinylated mouse-anti-ChIFN-γ and streptavidin-alkaline phosphatase. The assay was developed using BCIP/NBT substrate, and analyzed using the A·EL·VIS machine and the Eli.Analyse software (Version 4.0) that allows for automated counting of the number of spots based on size and intensity. The cow PBMC ELISPOT was performed as described by Koets et al. (2006).

2.4. ELISA for ChIFN-γ detection

A total of 2 x 10^6 splenocytes per chicken were incubated for 48 h in a 24-wells plate in 500 µl of culture medium at 41°C, 5% CO_2, without stimulation or in the presence of ConA. Afterwards, supernatants were tested for ChIFN-γ using a commercial sandwich ELISA kit (Biosource International, California, USA) according to manufacturer's instructions.

2.5. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples isolated from spleen were screened for mRNA encoding ChIFN-γ, as described in Ariaans et al. (2008a). Briefly, total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Reverse transcription was performed using iScript cDNA Synthesis Kit (Biorad). The forward primer (5'-GTGAAGAAGGTGAAAGATATCATGGA-3') and reverse primer (5'- GCTTTGCGCTGGATTCTCA-3') and the probe (5'-FAM-TGGCCAAGCTCCCGATGAACGA-TAMRA-3') for ChIFN-γ were designed according to Kaiser et al. (2003). Amplification and detection of specific products was achieved with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Results were expressed as fold change between samples (Philbin et al., 2005).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 for Windows. Analysis of data was performed using a Mann-Whitney test. Results were considered statistically significant if $p \leq 0.05$.

3. Results

White Leghorn chickens were inoculated with virulent IBV strain M41 and at four different time points after inoculation spleens were harvested. Splenocytes of IBV-infected and uninfected chickens were incubated for 48 h with ConA, PMA and ionomycin, and different coronaviruses. ChIFN-γ production increased significantly after *in vitro* restimulation with IBV in both the IBV-infected and uninfected chickens (figure 1). Two mammalian coronaviruses did not cause an increase in ChIFN-γ production, indicating that the observed effect was specific for IBV and not for coronaviruses in general.

To determine if the IBV-induced ChIFN-γ production was caused by virus invasion and replication or an exclusively cell-mediated response to IBV, the virus was inactivated by exposure to ultraviolet light (figure 1, 13 dpi). ChIFN-γ production after exposure to the inactivated IBV did decrease in the IBV-uninfected birds when compared to IBV-infected birds, but it was still higher than in unstimulated cells.

Although a decrease in ChIFN-γ production was detected after UV-inactivation of the IBV, it cannot be excluded that the immune response in the IBV-infected chickens had already passed its peak at this time point. The experiment was repeated with IBV and with another chicken virus, NDV, both live and UV-

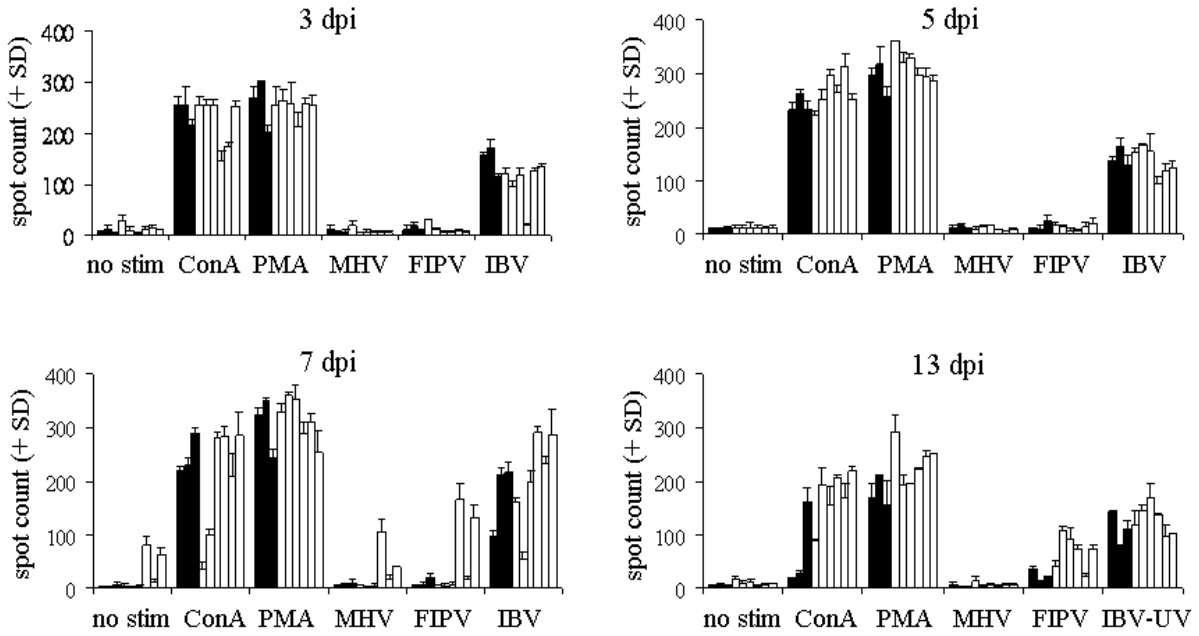

Figure 1. *ELISPOT assay to measure ChIFN-γ production by splenocytes of IBV-uninfected (black bars; n=3) versus IBV M41-infected (white bars; n=6) chickens at 3, 5, 7, or 13 days post-infection. Splenocytes were added to ChIFN-γ-coated wells and stimulated for 48 h with medium, ConA (10 µg/ml), PMA (100 ng/ml) and ionomycin (500 ng/ml), MHV (10^6 pfu), FIPV (10^6 pfu), IBV M41 (10^5 EID_{50}), or UV-inactivated IBV M41 (IBV-UV; 10^5 EID_{50}). Spot counts represent the number of ChIFN-γ secreting cells. Each bar represents the average of three samples per chicken, plus standard deviation.*

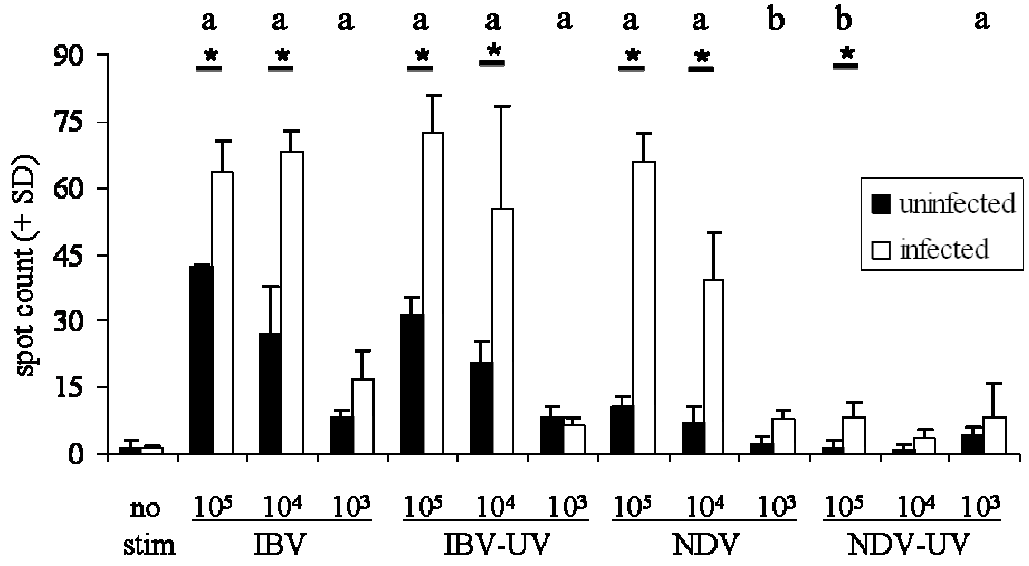

Figure 2. *ELISPOT assay to measure ChIFN-γ. Splenocytes of an uninfected (black bars) and an IBV- and NDV-vaccinated White Leghorn chicken (white bars) were stimulated in triplicate with three different doses of untreated or UV-inactivated IBV M41 or NDV. Bars marked with * show a significant difference ($p < 0.05$) between infected and uninfected birds. Bars marked with 'a' show a significant difference between the infected and uninfected birds on the one hand and the unstimulated controls on the other. Bars marked with 'b' show a significant difference between infected birds and unstimulated controls.*

inactivated. Splenocytes of an uninfected chicken and a chicken that had been vaccinated for both IBV and NDV were restimulated *in vitro* with different concentrations of either virulent or UV-inactivated IBV M41 or NDV (figure 2).

Only the splenocytes from the NDV-vaccinated chicken produced ChIFN-γ after NDV restimulation, and hardly any response to the UV-inactivated virus was detected. In contrast, both the IBV-vaccinated and the uninfected chicken showed a dose-dependent response to restimulation with IBV, with no discernable difference between treated and untreated virus in the infected chicken. Stimulation with UV-inactivated IBV resulted in decreased ChIFN-γ production in the uninfected chicken compared to the infected chicken, but the response was still elevated compared to the unstimulated control.

To test if the observed effect is a specific interaction between the virus and its natural host, or if IBV can activate leukocytes regardless of the species from which the cells were obtained, we stimulated chicken and cow leukocytes with mitogens and different avian viruses (figure 3). Splenocytes of an IBV- and NDV-vaccinated chicken produced ChIFN-γ after incubation with IBV and UV-inactivated IBV, whereas cow PBMC did not show any response. A third poultry virus, REV, did not induce IFN-γ. These results demonstrate that the immune activation by IBV is species-specific, and that other poultry viruses do not cause a similar rapid immune activation upon first exposure.

IBV is known to induce rapid production of type-I IFNs by chicken leukocytes in both naïve and IBV-infected chickens (Pei et al., 2001). Type-I IFNs are heat-stable, whereas IFN-γ is heat-sensitive. To exclude that the anti-IFN-γ antibodies used in our ELISPOT assay are cross-reacting with type-I IFNs or other heat-stable antigens produced by IBV-infected cells, we tested the effect of heat inactivation. Chicken splenocytes were cultured for 48 h in the presence of different stimuli, the supernatants were collected and part of the samples heat-inactivated at 65°C for 30

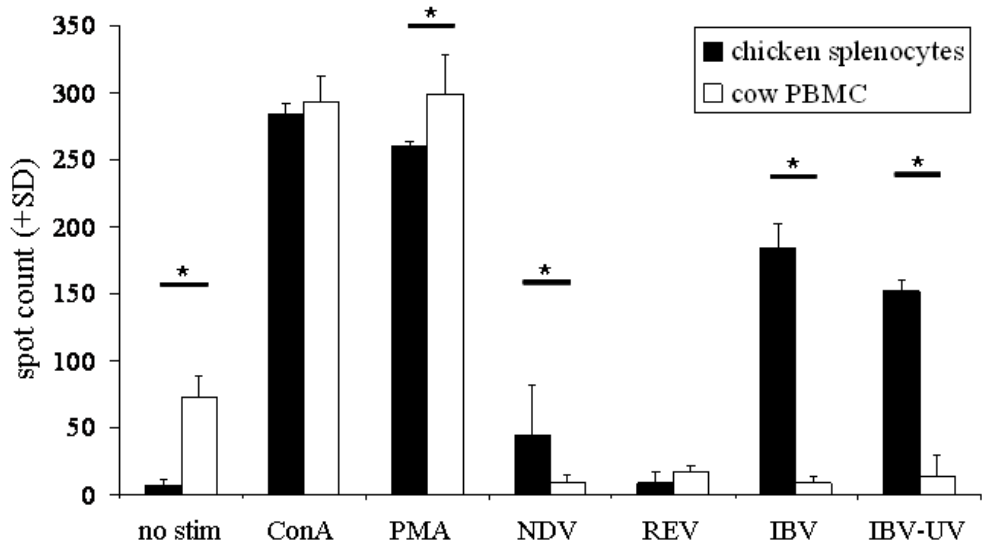

Figure 3. *ELISPOT assay to measure the production of IFN-γ by White Leghorn chicken splenocytes (black bars) and cow PBMC (white bars) in triplicate after in vitro stimulation with ConA (10 µg/ml), PMA (100 ng/ml) and ionomycin (500 ng/ml), NDV (10^5 EID_{50}), REV (10^5 EID_{50}), IBV M41 (10^5 EID_{50}), or UV-inactivated IBV M41 (10^5 EID_{50}). Bars marked with * are significantly different ($p < 0.05$).*

min. We tested all the samples using a commercial ELISA for chIFN-γ detection (figure 4). After heat-inactivation, ChIFN-γ levels in the supernatants decreased significantly when compared to untreated supernatants, confirming that the observed signal was indeed specific for ChIFN-γ and not due to cross-reactivity with type-I IFNs or other heat-stable proteins.

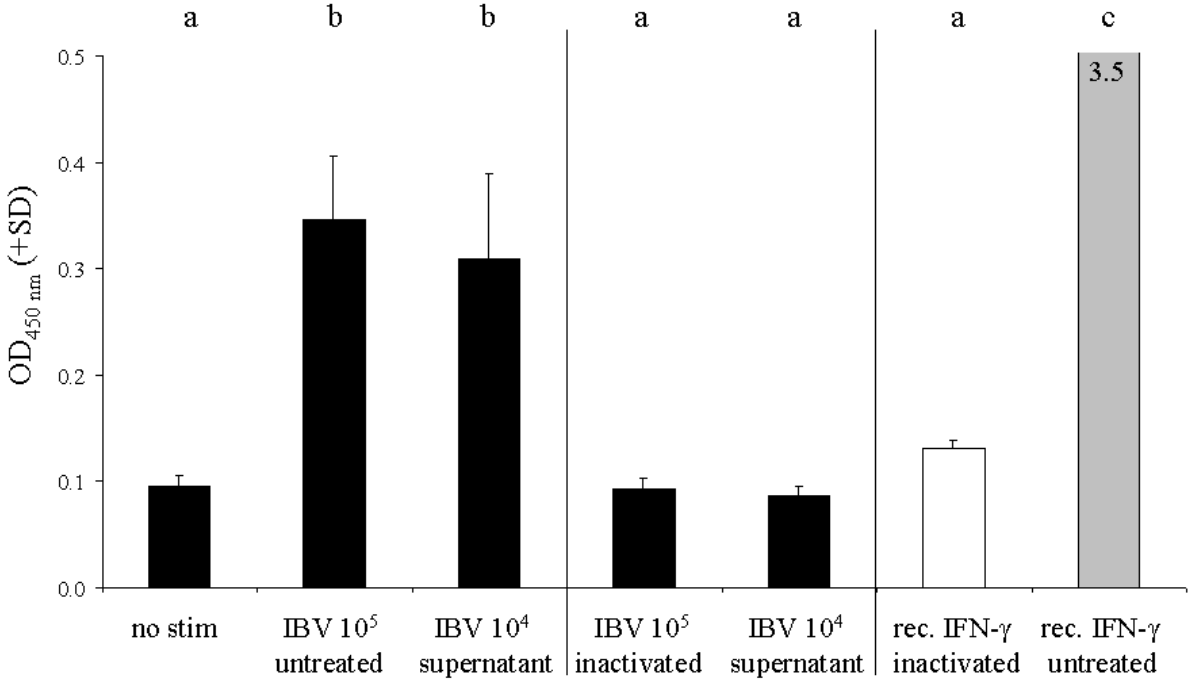

Figure 4. *Chicken splenocytes were incubated in triplicate for 48 h with medium or IBV M41 (10^5 and 10^4 EID_{50}). After 48 h, culture supernatants were collected and half of the samples were incubated at 65°C for 30 min. Supernatants were tested using a commercial ELISA for ChIFN-γ. Recombinant ChIFN-γ protein (10 ng/ml) with or without heat treatment was included as a control. Groups with different letters are significantly different ($p < 0.05$).*

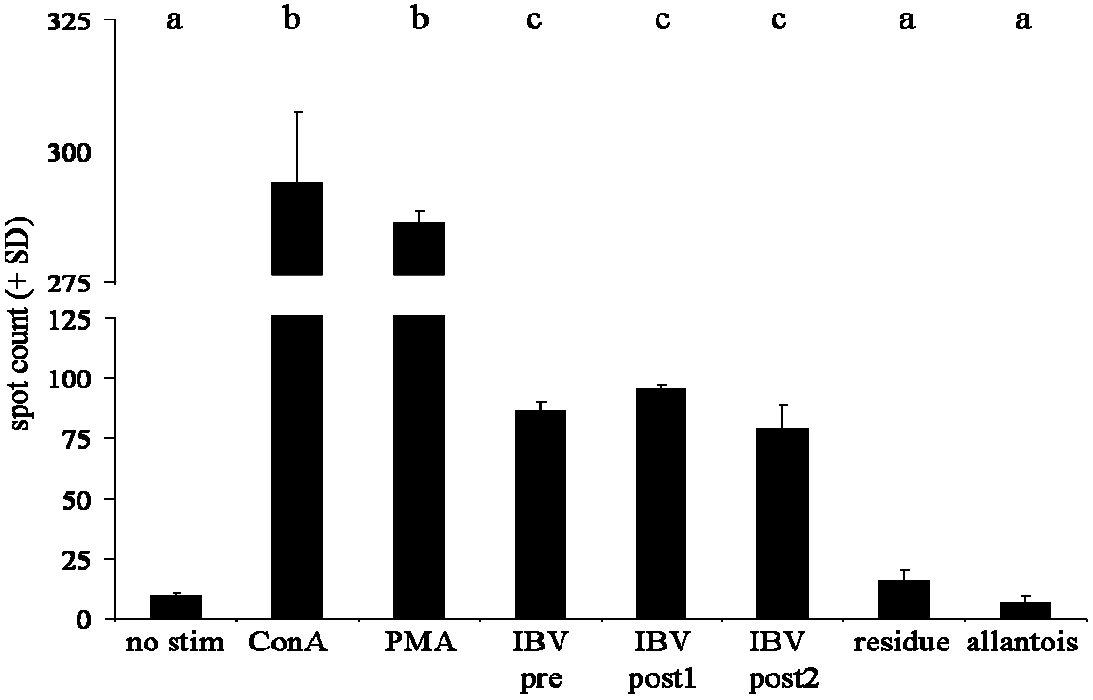

Figure 5. *ELISPOT for detection of ChIFN-γ produced by splenocytes of 4 chickens after 24 h incubation with the following stimuli: culture medium, ConA (10 µg/ml), PMA (100 ng/ml) and ionomycin (500 ng/ml), 10^5 EID_{50} IBV M41 before (IBV pre) sucrose purification, IBV M41 pooled from the 3 IBV-richest sucrose fractions (IBV post1, ~10^5 EID_{50}) and pooled from 3 surrounding fractions (IBV post2, ~10^5 EID_{50}), a pool of 3 sucrose fractions outside the IBV band (residue, same volume as 'post2' sucrose fraction), and allantois from an IBV-uninfected egg. Groups with different letters are significantly different ($p < 0.05$).*

To ensure that the induction of ChIFN-γ was not due to contaminating components derived from the allantois fluid in which the virus was grown or from the production process, the virus was purified with a sucrose gradient and tested in an ELISPOT assay. Splenocytes from four IBV-uninfected chickens were stimulated with purified and unpurified IBV (figure 5). A fraction of the sucrose gradient obtained from outside the IBV band and allantois fluid from an IBV-uninfected egg (10x diluted in PBS) were also included. All splenocyte cultures showed a significant increase in ChIFN-γ production after stimulation with IBV M41 before and after sucrose-purification, with no significant differences between them. Incubation with a fraction of the sucrose gradient obtained from outside the IBV band ('residue') and with IBV-free allantois fluid did show any effect. We therefore conclude that the increase in ChIFN-γ production is caused by the IBV itself and not by other components.

To study the kinetics with which IFN-γ was induced, we performed quantitative RT-PCR (qPCR) for detection of ChIFN-γ mRNA (figure 6 and 7). An increase in ChIFN-γ mRNA expression was detected as early as 2 h after stimulation with virulent IBV M41 and with IBV vaccine strain H120, in splenocytes of layer birds as wells as splenocytes of broilers.

4. Discussion

IFN-α and IFN-β, both type-I interferons, are important in the innate immune defence against virus infection and are quickly upregulated after infection with

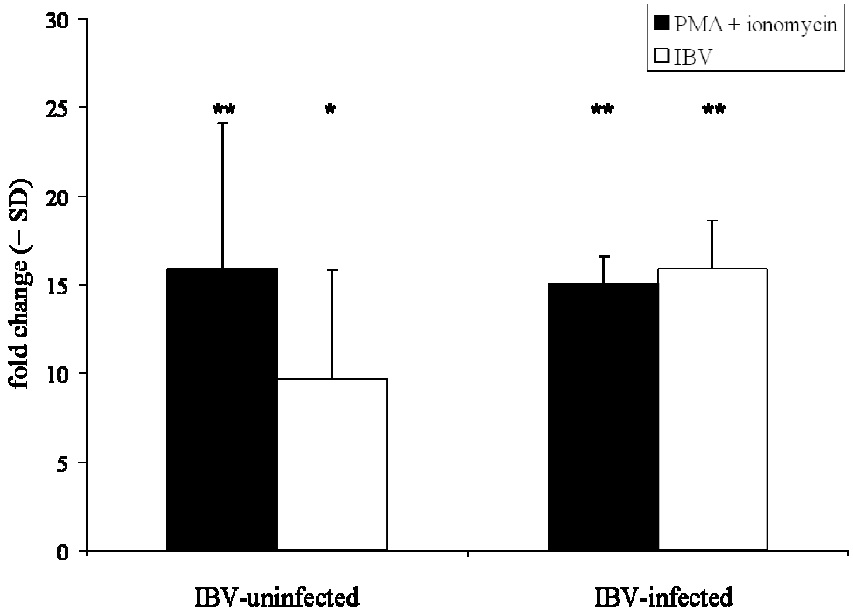

Figure 6. *Real-time quantification of ChIFN-γ mRNA expression by splenocytes from four IBV-infected and four IBV-uninfected commercial broiler chickens, 6 h after stimulation with PMA (100 ng/ml) and ionomycin (500 ng/ml), or IBV M41 (10^5 EID_{50}). Bars represent the mean fold increase (plus standard deviation) compared to unstimulated splenocytes. All groups are significantly different from the unstimulated splenocytes (*: $p < 0.05$; **: $p < 0.01$).*

viruses (Kawai and Akira, 2006). IFN-γ, a type-II interferon, is associated with adaptive immune responses and as such would be expected to be upregulated later in the immune response (Takaoka and Yanai, 2006). In contrast, we observed a rapid increase in ChIFN-γ production within 24 h after stimulation of chicken splenocytes with coronavirus IBV strain M41, and an increased expression of ChIFN-γ mRNA as early as 1 hour after stimulation. This increase was detected not only in *in vitro* restimulated splenocytes of chickens that had previously been exposed to IBV, but also in splenocytes of IBV-naïve chickens. Furthermore, two chicken viruses that are not members of the Coronaviridae family, the avian paramyxovirus NDV and the retrovirus REV, did not induce ChIFN-γ production in splenocytes of uninfected chickens. IBV did not induce production of IFN-γ by leukocytes of mammalian origin, cow PBMC. This indicates that the observed IFN-γ production appears to be a specific interaction between IBV and its natural host, and not an effect generally observed after exposure of non-avian leukocytes to IBV, or of chicken leukocytes to non-avian coronaviruses or to other avian viruses.

To ensure that the observed response was indeed due to ChIFN-γ detection and not to type-I interferons, samples were heated to 65°C after incubation. This inactivates IFN-γ but not type-I interferons (Lowenthal et al., 2001). After heat-treatment, the concentration of IFN-γ protein in the IBV-stimulated samples dropped to background levels, indicating that the observed response is indeed ChIFN-γ-specific and not due to type-I interferons.

In virus taxonomy, coronaviruses are currently assigned into four different groups, with groups 1 and 2 consisting of various mammalian coronaviruses, group 3 formed exclusively by avian coronaviruses such as IBV, Turkey coronavirus (TCoV) and Pheasant coronavirus, and group 4 currently only containing the

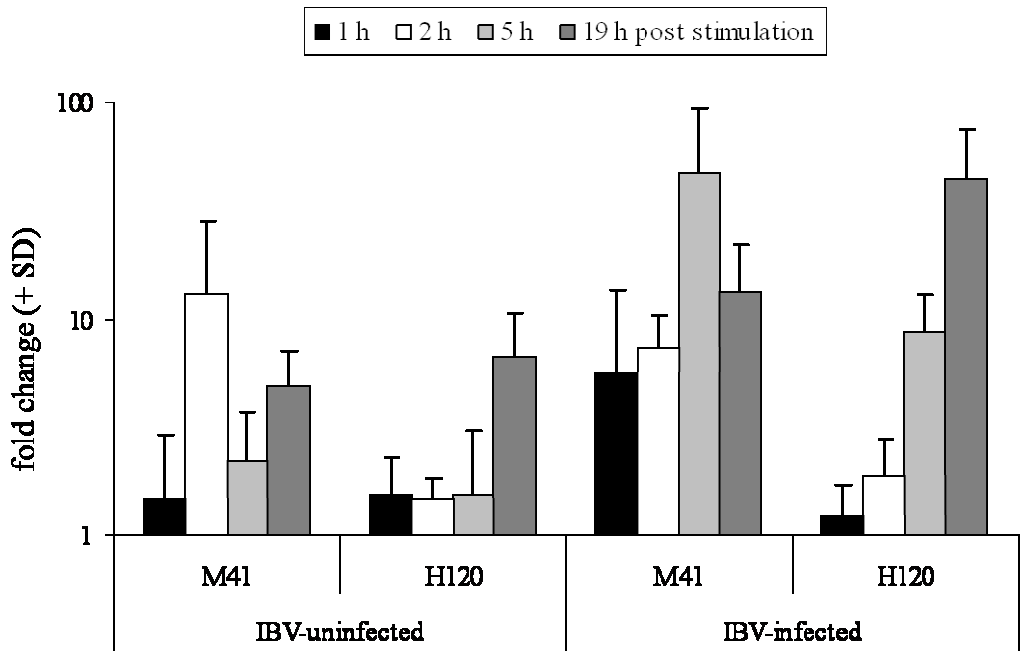

Figure 7. *Real-time quantification of ChIFN-γ mRNA expression by splenocytes from four IBV-infected chickens, at four different time points after stimulation with PMA (100 ng/ml) and ionomycin (500 ng / ml), IBV M41 (10^5 EID_{50}), or IBV H120 (10^5 EID_{50}). Bars represent the mean fold increase (plus standard deviation) compared to unstimulated splenocytes of four IBV-uninfected birds.*

recently discovered human coronavirus SARS-CoV (Cavanagh, 2007). Chicken splenocytes were *in vitro* stimulated with members of the other corononavirus groups that have mammalian hosts, the group 1 coronavirus FIPV and group 2 coronavirus MHV. Both viruses did not induce ChIFN-γ production by chicken splenocytes. For MHV several studies have shown a clear difference in IFN-γ production between infected and uninfected mice by ELISA and qPCR (Hooks et al., 2003; Lucchiari et al., 1991). Elevated levels of IFN-γ mRNA were detected in a pilot study with a FIPV-infected cat when compared to uninfected cats (Kiss et al., 2004).

Castilletti et al. (2005) showed that the group 4 human coronavirus SARS-CoV, which is thought to be closely related to IBV, induced both IFN-α and IFN-γ mRNA and protein after stimulation of PBMC from healthy donors in a dose-dependent manner within 24 h, without the need for viral replication. They suggest that exposure of PBMC to high virus titers *in vivo* at the site of infection is biologically likely and as such concomitant activation of IFN-α and IFN-γ after first-time exposure to relatively high levels of coronavirus might be relevant to the pathogenesis of the disease. A comparison between PBMC of healthy donors and SARS patients was not made, and it therefore remains unknown whether the kinetics of IFN-γ production after exposure to SARS-CoV is similar in SARS patients and healthy donors.

Both IBV and SARS-CoV infect and replicate in the respiratory tract, and have been reported to spread to other enteric tissues such as the gastrointestinal tract (Cavanagh, 2007; Navas-Martin and Weiss, 2004). Infection with these viruses leads to leukocyte influx and damage of respiratory tract epithelium. Matthijs et al. (accepted 2008 Vet. Immunol. Immunopath.) found very high numbers of macrophages and $CD4^+$ T cells in trachea and lung of IBV H120 and M41 infected

birds. The local immune deregulation might in turn give rise to enhanced susceptibility to secondary bacterial infections, such as colibacillosis in poultry after IBV infection, or to organ destruction and pneumonia-like symptoms in the case of SARS. Interestingly, highly elevated levels of IFN-γ and related cytokines and chemokines, a so-called “cytokine storm”, were found already at the day of fever onset in SARS patients (Huang et al., 2005), and improperly modulated IFN type I and II responses are thought to be closely linked to the clinical course of SARS (Cameron et al., 2007). These elevated cytokine and chemokine levels could be one of the causes of the observed leukocyte influx in the lungs and this in turn could result in the organ destruction and immune system exhaustion that are a hallmark of SARS. Unfortunately, the cytokine kinetics in the SARS study were examined at a later time interval than in our study and can therefore not support or disprove the possibility of SARS-CoV acting as a polyclonal stimulus.

In conclusion, we have shown a rapid increase in ChIFN-γ production by chicken leukocytes after *in vitro* stimulation with IBV. This increase is independent of the infection status of the chicken and appears to be IBV-specific, as we did not observe this effect with other chicken viruses, other coronaviruses or components from allantoic fluid from which the virus was isolated. The observed effect suggests that IBV acts as a polyclonal stimulus on chicken immune cells.

Acknowledgements

We thank Daphne van Haarlem for technical assistance. This work was supported by BSIK VIRGO consortium grant (G 03012), the Netherlands to P.M.H. and L.V..

References

Ariaans, M.P., Matthijs, M.G.R., Van Haarlem, D., Van de Haar, P., Van Eck, J.H.H., Hensen, E.J., Vervelde, L., 2008a. The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after Infectious Bronchitis Virus infection. Vet. Immunol. Immunopathol. 123, 240-250.

Ariaans, M.P., Van de Haar, P.M., Lowenthal, J.W., Van Eden, W., Hensen, E.J., Vervelde, L., 2008b. ELISPOT and intracellular cytokine staining: novel assays for quantifying T cell responses in the chicken. Dev. Comp. Immunol. 32, 1398-1404.

Cameron, M. J., Ran, L., Xu, L., Danesh, A., Bermejo-Martin, J. F., Cameron, C. M., Muller, M. P., Gold, W. L., Richardson, S. E., Poutanen, S. M., Willey, B. M., DeVries, M. E., Fang, Y., Seneviratne, C., Bosinger, S. E., Persad, D., Wilkinson, P., Greller, L. D., Somogyi, R., Humar, A., Keshavjee, S., Louie, M., Loeb, M. B., Brunton, J., McGeer, A. J., and Kelvin, D. J., 2007. Interferon-mediated immunopathological events are associated with atypical innate and adaptive immune responses in patients with severe acute respiratory syndrome. J. Virol. 81, 8692-8706.

Castilletti, C., Bordi, L., Lalle, E., Rozera, G., Poccia, F., Agrati, C., Abbate, I., and Capobianchi, M. R., 2005. Coordinate induction of IFN-alpha and -gamma by SARS-CoV also in the absence of virus replication. Virology 341, 163-169.

Cavanagh, D., 2003. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol. 32, 567-582.

Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. Vet. Res. 38,

281-297.
Collisson, E. W., Pei, J., Dzielawa, J., and Seo, S. H., 2000. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev. Comp. Immunol. 24, 187-200.
Cornelissen, L. A., Wierda, C. M., van der Meer, F. J., Herrewegh, A. A., Horzinek, M. C., Egberink, H. F., and de Groot, R. J., 1997. Hemagglutinin-esterase, a novel structural protein of torovirus. J. Virol. 71, 5277-5286.
Hackney, K., Cavanagh, D., Kaiser, P., and Britton, P., 2003. In vitro and in ovo expression of chicken gamma interferon by a defective RNA of avian coronavirus infectious bronchitis virus. J. Virol. 77, 5694-5702.
Hooks, J. J., Wang, Y., and Detrick, B., 2003. The critical role of IFN-gamma in experimental coronavirus retinopathy. Invest. Ophthalmol. Vis. Sci. 44, 3402-3408.
Huang, K. J., Su, I. J., Theron, M., Wu, Y. C., Lai, S. K., Liu, C. C., and Lei, H. Y., 2005. An interferon-gamma-related cytokine storm in SARS patients. J. Med. Virol. 75, 185-194.
Kaiser, P., Underwood, G., and Davison, F., 2003. Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. J. Virol. 77, 762-768.
Kawai, T., and Akira, S., 2006. Innate immune recognition of viral infection. Nat. Immunol. 7, 131-137.
Kiss, I., Poland, A. M., and Pedersen, N. C., 2004. Disease outcome and cytokine responses in cats immunized with an avirulent feline infectious peritonitis virus (FIPV)-UCD1 and challenge-exposed with virulent FIPV-UCD8. J. Feline Med. Surg. 6, 89-97.
Koets, A., Hoek, A., Langelaar, M., Overdijk, M., Santema, W., Franken, P., Eden, W., and Rutten, V., 2006. Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. Vaccine 24, 2550-2559.
Koopmans, M., Ederveen, J., Woode, G. N., and Horzinek, M. C., 1986. Surface proteins of Breda virus. Am. J. Vet. Res. 47, 1896-1900.
Lowenthal, J. W., Staeheli, P., Schultz, U., Sekellick, M. J., and Marcus, P. I., 2001. Nomenclature of avian interferon proteins. J. Interf. Cytok. Res. 21, 547-549.
Lucchiari, M. A., Martin, J. P., Modolell, M., and Pereira, C. A., 1991. Acquired immunity of A/J mice to mouse hepatitis virus 3 infection: dependence on interferon-gamma synthesis and macrophage sensitivity to interferon-gamma. J. Gen. Virol. 72 , 1317-1322.
Matthijs, M.G.R., Ariaans, M.P., Dwars, R.M., Van Eck, J.H.H., Bouma, A., Stegeman, A., Vervelde, L., 2008. Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with *E. coli*. Vet. Immunol. Immunopathol., accepted for publication.
Navas-Martin, S. R., and Weiss, S., 2004. Coronavirus replication and pathogenesis: Implications for the recent outbreak of severe acute respiratory syndrome (SARS), and the challenge for vaccine development. J. Neurovirol. 10, 75-85.
Otsuki, K., Nakamura, T., Kawaoka, Y., and Tsubokura, M., 1988. Interferon induction by several strains of avian infectious bronchitis virus, a coronavirus, in chickens. Acta Virol. 32, 55-59.
Pei, J., Sekellick, M. J., Marcus, P. I., Choi, I. S., and Collisson, E. W., 2001. Chicken interferon type I inhibits infectious bronchitis virus replication and associated respiratory illness. J. Interf. Cytok. Res. 21, 1071-1077.
Philbin, V. J., Iqbal, M., Boyd, Y., Goodchild, M. J., Beal, R. K., Bumstead, N.,

Young, J., and Smith, A. L., 2005. Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. Immunology 114, 507-521.
Ravindra, P. V., Tiwari, A. K., Ratta, B., Chaturvedi, U., Palia, S. K., Subudhi, P. K., Kumar, R., Sharma, B., Rai, A., and Chauhan, R. S., 2008. Induction of apoptosis in Vero cells by Newcastle disease virus requires viral replication, de-novo protein synthesis and caspase activation. Virus Res. 133, 285-290.
Stern, D. F., and Sefton, B. M., 1982. Synthesis of coronavirus mRNAs: kinetics of inactivation of infectious bronchitis virus RNA synthesis by UV light. J. Virol. 42, 755-759.
Takaoka, A., and Yanai, H., 2006. Interferon signalling network in innate defence. Cell Microbiol. 8, 907-922.

CHAPTER 4

DNA immunization as a tool to study T-cell responses and induce protection against Infectious Bronchitis Virus

Mark Ariaans[1], Ines Pereira[1], Robbert van der Most[1], Evert Hensen[1], Lonneke Vervelde[1]

[1]Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

Manuscript in preparation

Abstract

We investigated whether immunization with a DNA plasmid encoding the Infectious Bronchitis Virus nucleocapsid protein protected chickens against a challenge with virulent IBV M41. Our aim was to test an immunogenic carrier system that potentially enables us to express and present various antigens of limited complexity, in order to measure T cell responses under standardized conditions for different antigenic determinants. Birds that had been treated with the plasmids encoding the IBV nucleocapsid protein showed less severe clinical signs than those treated with control plasmid. No significant differences were found in the level of nucleocapsid-specific IgM and IgG antibodies in the sera of IBV-infected chickens with or without nucleocapsid plasmid immunization. IFN-γ production after in vitro stimulation was used as the first choice to measure T-cell reactivity. Surprisingly, splenocytes of chickens in all groups showed significantly increased IFN-γ production after in vitro stimulation with IBV, regardless of prior immunization.

Key words: Infectious Bronchitis Virus, DNA immunization, nucleocapsid, chicken, vaccination

1. Introduction

Cytotoxicity and / or T cell help are a necessity for successful protection against a range of poultry pathogens such as Infectious Bronchitis Virus (IBV; Seo and Collisson, 1998), Avian Leukosis Virus (ALV; Thacker et al., 1995) and Reticulo-endotheliosis Virus (REV; Weinstock et al., 1989). Many poultry vaccines induce a good humoral response, but little is known about the contribution of a cell-mediated response. These vaccines often consist of live attenuated or killed virus, which likely result in completely different types of cell-mediated responses.

The aim of our study was to develop an immunogenic carrier system, in which at a later stage different antigenic determinants can be exchanged to test one specific determinant at a time under comparable conditions. This allows us to test the T cell response in more detail and to evaluate the contribution of $CD4^+$ or $CD8^+$ T cells independently. Immunization with purified proteins has been shown inefficient in producing cell-mediated cytotoxicity ($CD8^+$ T-cell), because it favours presentation to $CD4^+$ T cells via MHC class II molecules (Barrett/Rezvani, 2007; Machiels et al, 2002; Rammensee et al, 1996).

Peptides selected for their MHC specific binding in the recipient have been tested and shown effective in some species for MHC class I and class II restricted responses. Hofmann et al. (2003) showed that chickens immunized four times with a liposome-entrapped synthetic peptide from Rous sarcoma virus, selected for its stringent binding with chicken MHC (B) class I (B-F) of an inbred chicken line B^{12}, were partially protected against tumour induction. A peptide-binding motif for MHC class II (B-L) of inbred chicken line B^{19} (P2a) has also been described recently (Cumberbatch et al., 2006), potentially allowing the generation of class II specific peptides.

DNA plasmids encoding specific antigens do elicit both humoral and cell-mediated immune responses and induce protective immunity against a range of infectious diseases such as Infectious Bursal Disease (Li et al., 2003), Marek's Disease (Tischer et al., 2002) and duck Hepatitis B (Miller et al., 2006). Efficient induction of immune responses has been observed using this method, despite minimal production of the actual antigen (usually in the pico- to nanogram range). This could partly be contributed to the adjuvant effect of the viral DNA, which induces innate immune responses to non-methylated CpG motifs (Oshop et al., 2002; Stevenson and Riley, 2004).

Infectious Bronchitis Virus (IBV) is a highly contagious respiratory virus in poultry. Some IBV strains also affect the reproductive system and the kidneys. IBV is considered a major cause of economic loss to the poultry industry worldwide (Ignjatovic and Sapats, 2000). The virus belongs to the Coronaviridae family and consists of a lipid membrane, a single-stranded RNA genome and three major structural proteins: the genome-associated internal nucleocapsid (N) protein and the matrix (M) and spike (S) membrane proteins. Although the S protein is the major inducer of neutralizing antibodies in IBV, it is highly variable between IBV strains. The N protein (IBV_N) on the other hand is highly conserved between strains (Cavanagh, 2003; Williams et al., 1992), which makes it an attractive vaccine candidate. Furthermore, IBV_N has been shown to induce cytotoxic T lymphocyte immunity in chickens (Seo et al., 1997).

In this paper we study the protective capacity of a modified Semliki Forest Virus (SFV) plasmid (DiCiommo and Bremner, 1998) expressing IBV_N on a challenge infection with virulent IBV strain M41. The plasmid contains a CMV

promotor and an SV40 terminator, which makes it functional for *in vivo* use in most vertebrates including chickens (Lee et al., 1999; Suarez and Schultz-Cherry, 2000). The resulting vector is a self-replicating RNA which does not create virus particles, for it lacks the genes encoding the capsid proteins. Replication of the plasmid leads to induction of apoptosis in the host cell, thereby eliminating the chance of uncontrolled spread to neighbouring cells and improving uptake by dendritic cells (Albert et al., 1998). The DNA plasmid acts as an adjuvant, facilitating presentation by dendritic cells of IBV_N via MHC class I and II molecules, which results in the activation of both cell-mediated and humoral immune responses. The vector contains a subgenomic promotor upstream of the intended IBV_N gene insertion site, which induces enhanced production of IBV_N protein.

To determine the clinical and immunological effects of the DNA immunization, all chickens were monitored for clinical signs of IBV infection, IBV specific antibody production and IFN-γ production.

2. Materials and methods

2.1. Virus

Massachusetts IBV M41 virus (1st egg passage) was obtained from the Animal Health Service (GD Deventer, The Netherlands) as commercial freeze-dried vials at $10^{7.3}$ EID_{50} per 0.2 ml. IBV inocula were prepared in phosphate-buffered saline (PBS, <1.0 EU/ml endotoxin; Cambrex) just before use.

2.2. Amplification and cloning of IBV nucleocapsid gene

Primers were designed to the 5' end (containing BamHI restriction site; 5'-CG**GGATCC**GCAAGCGGTAAGGCAACTGG-3') and 3' end (containing XmaI restriction site; 5'-AGGG**CCCGGG**AAGTTCATTCTCTCCTAGAG-3') of the complete IBV M41 nucleocapsid (henceforth designated as IBV_N) gene and used to amplify the gene. The PCR product was cloned into a pCR2.1-TOPO® vector (Invitrogen), propagated in *E. coli* DH5α and sequenced (BaseClear, The Netherlands).

2.3. Construction of immunization plasmids

The IBV_N gene was cut from the pCR2.1-TOPO® vector using the BamHI and XmaI restriction enzymes and cloned into a modified Semliki Forest Virus plasmid designated pSMART2a (a gift from dr. R. Bremner; DiCiommo and Bremner, 1998). The gene was inserted downstream of a FLAG and a 10xHis tag (figure 1). Correct

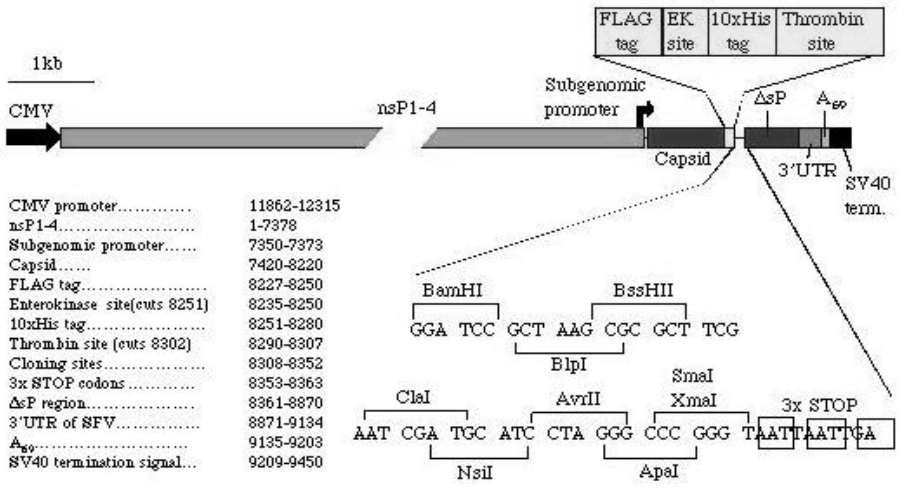

Figure 1. *Schematic representation of pSMART2a plasmid.*

insertion into the plasmid was confirmed by sequence analysis. The control plasmid consisted of a similarly modified SFV plasmid expressing the LacZ gene (kindly provided by dr. E. Tijhaar).

The plasmids were replicated in *E. coli* DH5α and isolated using the EndoFree Plasmid Mega Kit (QIAGEN) according to the manufacturer's protocol. Plasmids were stored at -20°C in TE buffer.

2.4. Immunofluorescent staining for detection of in vitro *protein production*

Protein expression was tested by immunofluorescent labeling of the FLAG tag protein linked to IBV_N. Baby Hamster Kidney (BHK-21) cells were cultured in DMEM (Gibco) with 5% FBS at 37°C, 5% CO_2. In brief, BHK-21 cells were transfected with the plasmid constructs using FuGENE^{TM} 6 Transfection Reagent (Roche Diagnostics) and incubated for 24 h at 37°C. Cells were fixed with 100% methanol and incubated for 1 h with mouse anti-FLAG antibody M2 (Sigma), followed by goat anti-mouse-FITC (Sigma).

2.5. Animals

White Leghorn chicken lines P2a ($B^{19}B^{19}$) and N2a ($B^{21}B^{21}$) were obtained at less than 1 week of age and housed in groups in an accredited isolation facility at Utrecht University. Chickens were fed *ad libitum* on commercial feed throughout the course of the experiments. All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

2.6. Experimental setup

Experiment 1. P2a and N2a chickens were randomly divided into 4 groups and individually tagged. All chickens received an intramuscular injection into the pectoral muscle at 7, 18 and 29 days of age. At each time point, serum samples were taken prior to injection of all chickens. Group 1 (n=10) received 3 injections with 30 µg pSMART2a-IBV_N plasmid in 50 µl PBS (<0.1 EU/ml endotoxin; Cambrex). Group 2 (n=8) received 2 injections with 30 µg pSMART2a-IBV_N followed by one mock injection with PBS. Group 3 (n=8) received 3 injections with 30 µg pSMART2a expressing the LacZ gene. Group 4 (n=7) received 3 mock injections with PBS. At 39 days of age, 10 days after the last immunization, all chickens were inoculated with 0.7 ml of 10^6 EID_{50}/ml IBV M41, intratracheally (0.5 ml) and by eye-nose drop (0.2 ml). At 46 days of age, 7 days post IBV-infection (dpi), 50% of the chickens in each group were killed and at 10 dpi the remaining chickens were killed. Serum, spleen and lung were sampled of each chicken for further analysis.

Experiment 2. P2a and N2a chickens were randomly divided into 3 groups of 10 chickens and individually tagged. All chickens received an intramuscular injection into the pectoral muscle at 7, 20 and 32 days of age. At each time point, serum samples were taken prior to injection of all chickens. Group 1 received 3 injections with 30 µg pSMART2a-IBV_N plasmid in 50 µl PBS. Group 2 received 2 injections with 30 µg pSMART2a-IBV_N followed by a mock injection with PBS. Group 3 received 3 injections with pSMART2a expressing the LacZ gene. At 40 days of age, all chickens were inoculated with 0.7 ml of 10^6 EID_{50}/ml IBV M41, intratracheally and by eye-nose drop. Five chickens from each group were killed at 7 and 10 dpi. Serum, spleen and lung were sampled of each chicken for further analysis.

2.7. Clinical observations

Chickens were scored on a daily basis for signs of clinical infection until 7 dpi, using the following scoring criteria: 0 = no clinical signs; 1 = very slight, incidental rales; 2 = sneezing/coughing and light, continuous rales; 3 = sneezing/coughing and clearly audible rales; 4 = dyspnea, gasping, loud rales (Pei et al., 2001). From 8 dpi onwards, clinical signs could not be detected. The clinical score of each chicken was defined as the total sum of daily scores from 1 to 7 dpi.

2.8. Serological detection of IBV antibodies

Serum samples of all birds were tested in triplicate for antibodies specific for IBV using a commercial IBV ELISA kit (BioChek) according to the manufacturer's protocol, in order to determine whether the infection was established.

Subsequently, serum samples of all birds were analysed in triplicate for antibodies specific for IBV nucleocapsid protein and β-galactosidase. Flatbottom 96-wells plates were coated ON with 100 ml/well of IBV_N (a kind gift of dr. J. Hiscox; Chen et al., 2003) or β-galactosidase (Calbiochem) protein at 0.5 mg/ml in carbonate-bicarbonate buffer (pH 9.7). Wells were blocked for 1 h with PBS with 1% milk powder, washed 4 times with PBS with 0.1% Tween-20, and incubated for 1 h with chicken sera diluted in assay buffer (PBS containing 1% milk powder and 0.1% Tween-20). As positive control, serum from an IBV-infected chicken (1:1000) and IBV_N specific antibodies (CVI-IBVN-48.4; ID Lelystad) were used. As a negative control, assay buffer instead of serum was included.

IBV_N-specific antibodies were detected using goat anti-chicken $IgG_{(H+L)}$-HRP in assay buffer, and stained with 100 ml 3,3',5,5'-tetramentylbenzidine (TMB) reagent (Pierce). Absorbance was measured within 30 min at l=450 nm.

2.9. ELISPOT for ChIFNg production

Spleen tissue was squeezed through a 70 µm gauze to prepare single cell suspensions. Splenocytes were isolated by density gradient centrifugation for 20 min at 850 x g using FICOLL-Hypaque (Sigma; density 1.078), washed twice with PBS and adjusted to 3 x 10^6 cells/ml in RPMI1640 culture medium containing 10% FBS, 2 mM glutamax-I (GIBCO) and 100 U/ml pen/strep (GIBCO). MultiScreen^{TM}-IP 96-well plates (MAIPS4510; Millipore) were coated with 5 µg/ml mouse anti-ChIFN-γ (CAC1233; Biosource International, California, USA) in coating buffer (sodium carbonate, 50 mM, pH 9.6) ON at 4°C. All incubation steps were performed with 100 µl/well. Plates were washed twice with blocking buffer (RPMI 1640 supplemented with 2% FCS, 2 mM glutamax-I, 100 U/ml P/S, 50 µM β-mercaptoethanol and incubated with blocking buffer for 1 h at 37°C, 5% CO_2. The blocking buffer was discarded and splenocytes were seeded at 3 x 10^5 cells/well in triplicate in culture medium. Cells were incubated in the presence of either culture medium or medium supplemented with one of the following stimuli to a final volume of 200 µl per well: 10 mg/ml ConcanavalinA (ConA; Sigma), 10 mg/ml β-galactosidase, 10 mg/ml IBV_N, 10 mg/ml pooled IBV spike or nucleocapsid peptides selected for their fit with the B^{19} MHC motif, 10^5 EID_{50} IBV M41, or 10^5 EID_{50} UV-inactivated IBV M41. The cells were incubated for 24 or 48 h at 41°C, 5% CO_2. Subsequently the plates were washed twice with distilled water, and three times with washing fluid (PBS supplemented with 0.1% Tween-20). ChIFN-γ was detected by incubation with 1 µg/ml biotinylated mouse-anti-ChIFN-γ (CAC1233; Biosource International) in assay buffer (PBS supplemented with 0.1% Tween-20 and 1% BSA; 100 µl/well) for 1 h at room temperature. Plates were washed four times with washing fluid and incubated

with 2 µg/ml streptavidin-alkaline phosphatase (Sigma; 100 µl/well) in assay buffer for 1 h at room temperature. Plates were washed five times with washing fluid and the assay was developed using BCIP/NBT substrate (Roche, Basel, Switzerland). The plates were then washed with copious amounts of tap water, air dried and analyzed using the A·EL·VIS machine and the Eli.Analyse software (Version 4.0) that allows for automated counting of the number of spots based on size and intensity.

2.10. Statistical analysis

Between-group differences were analyzed for each assay using one-way ANOVA. Analysis was performed using the SPSS program and the probability level for significance was taken as $P < 0.05$.

3. Results

3.1. IBV_N gene expression in BHK-21 cells

BHK-21 cells were transfected with the plasmids to determine whether protein was successfully produced. Cells were transfected with either empty pSMART2a plasmid or the pSMART2a-IBV_N plasmid. After transfection, protein production was visualized by indirect immunofluorescence using monoclonal antibodies directed against the protein-linked FLAG tag (figure 2). FLAG-specific staining was found in BHK-21 cells transfected with pSMART2a-IBV_N; cells transfected with the empty plasmid did not show any staining.

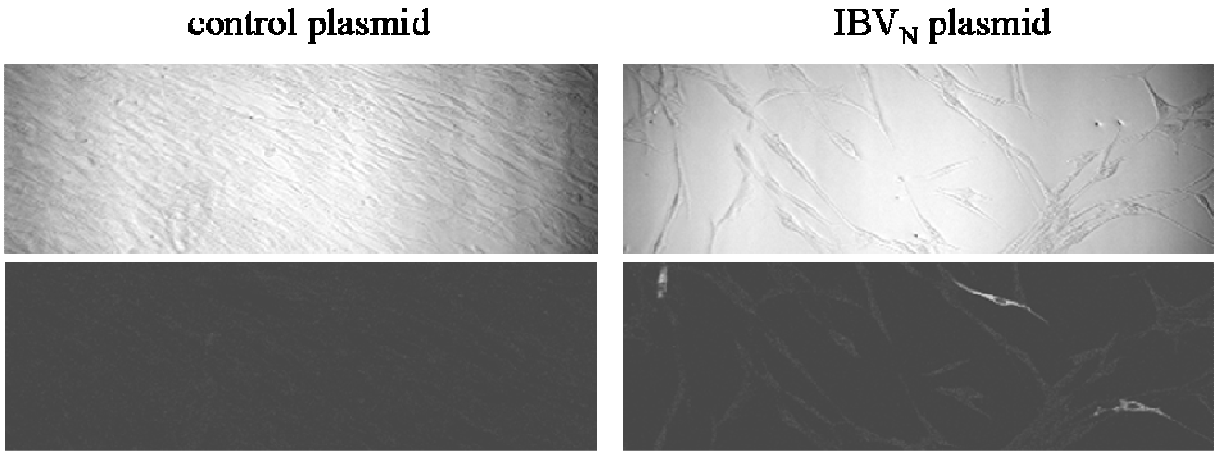

Figure 2. *Immunofluorescent staining of FLAG tags. BHK-21 cells were transfected with the pSMART2a plasmid construct expressing FLAG-tagged IBV_N protein. Cells transfected with an empty control plasmid are shown on the left (top: transmission image; bottom: fluorescence image). Cells expressing the IBV_N protein (right panels) show fluorescence after staining with anti-FLAG antibodies (bottom right).*

3.2. Clinical observations

In both experiments, all birds were challenged with virulent IBV M41 virus after two or three immunizations with plasmid. Chickens were monitored on a daily basis for clinical signs of IBV infection. The clinical score of each chicken was calculated by adding up the daily scores from the time of infection up to 7dpi. Between 7 and 10 dpi, clinical signs were not detected anymore in any bird.

In experiment 1, all PBS and pSMART2a-LacZ injected birds had high clinical

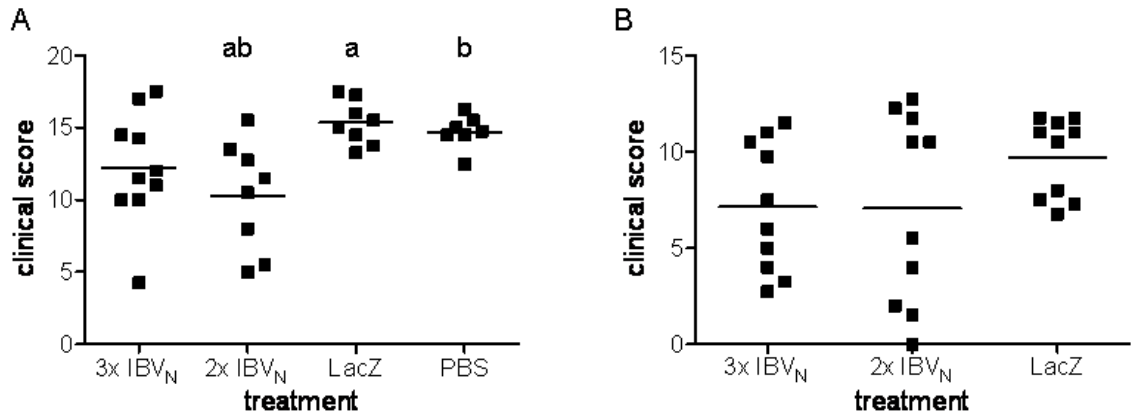

Figure 3. *Clinical score of IBV severity observed in chickens in experiment 1 (A) and 2 (B). Each dot represents the cumulative score of one chicken over 7 days of monitoring. Groups with corresponding letters are significantly different (P < 0.05).*

scores, whereas several birds who had received 2 or 3 injections with the pSMART2a-IBV$_N$ plasmid (2x IBV$_N$ and 3x IBV$_N$, respectively) had moderate to low clinical scores (figure 3A). The mean cumulative score of the 2x IBV$_N$ group was significantly lower (P<0.05) than that of the LacZ and PBS control groups, while the score of the 3x IBV$_N$ was markedly lower (P<0.1) than both control groups.

In experiment 2, all birds in the LacZ group had moderate to high clinical scores, whereas several birds in the 2x IBV$_N$ and 3x IBV$_N$ groups had low clinical scores (figure 3B). Both IBV$_N$ immunization groups had lower mean cumulative scores than the LacZ group, although these differences were not significant.

In both experiments the protective effect is more profound in the 2x IBV$_N$ group than in the 3x IBV$_N$ group, which suggests that a longer time interval between immunizations and virus challenge might be a more effective approach than an increased number of immunizations. No differences were observed between the two inbred chicken strains.

3.3. Serological detection of antibodies against IBV

To determine whether all chickens had been successfully challenged with IB virus, a commercial ELISA was used to detect antibodies against IBV (figure 4).

Using the commercial ELISA, in which wells are coated with whole virus particles, no antibodies to IBV could be detected after the immunizations but before IBV challenge in any of the groups. Ten days after IBV challenge, all birds had IBV-specific antibodies.

3.4. Serological detection of nucleocapsid-specific antibodies

To determine the ability of the IBV$_N$ plasmids to induce a humoral response, sera of all chickens were tested in ELISA for the presence of antibodies against IBV$_N$ protein and β-galactosidase. No antibodies against β-galactosidase were detected in any of the sera at all time points.

After DNA immunization and before IBV challenge, no antibodies were detected against the IBV$_N$ in the LacZ or PBS groups. A slight, although not significant, increase in IBV$_N$-specific antibody response was found in several chickens immunized two or three times with the IBV$_N$ plasmid when compared to the LacZ and PBS control groups (figure 5A). At 7 and 10 days after IBV infection (figure 5B), all chicken sera tested positive for antibodies against IBV$_N$, but with no

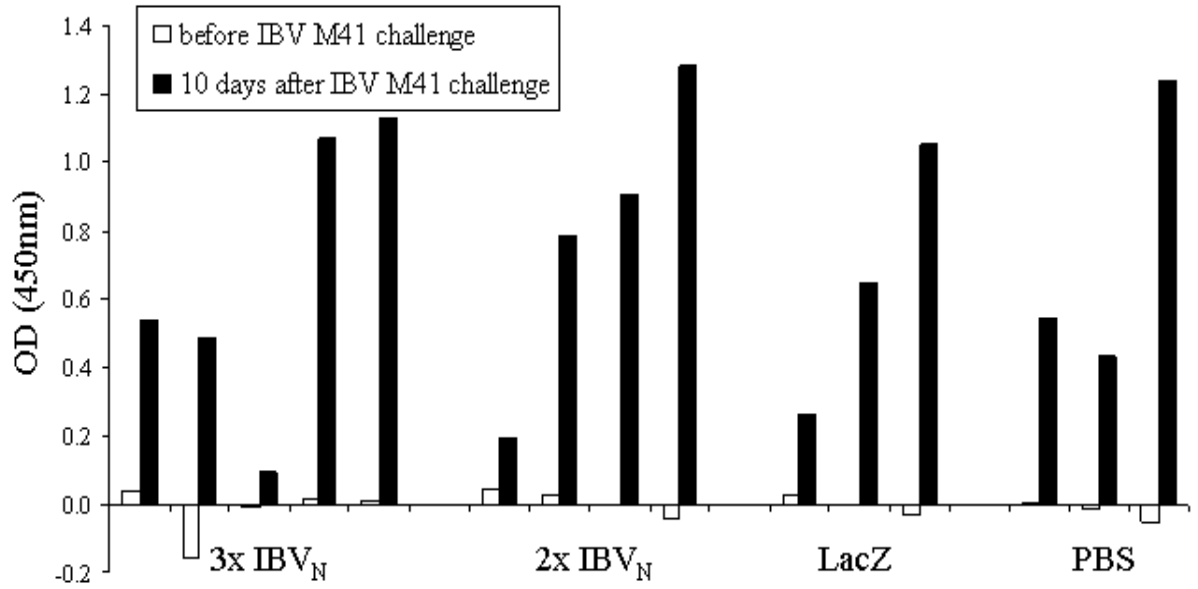

Figure 4. *Commercial ELISA for detection of antibodies against IB virus in 15 birds from experiment 1 after plasmid immunizations, before (white bars) and 10 days after (black bars) IBV M41 challenge. S/P values on the Y-axis are calculated as (sample mean – negative control mean) / (positive control mean – negative control mean).*

significant differences in magnitude between IBV_N-immunized and non-immunized chickens. Moreover, antibody titers did not correlate with clinical scores.

3.5. ELISPOT for IFN-γ production

To determine whether the plasmid immunizations induced T cell responses to the immunizing antigens, splenocytes were tested for their ability to produce IFN-γ in response to stimulation with IBV M41 or β-galactosidase (figure 6).

In experiment 1, no significant IFN-γ production was found after stimulation with β-galactosidase or the pooled spike (S) and nucleocapsid (N) peptides in any of the treatment groups and these stimulation conditions were dropped in the second experiment. In both experiments, all chickens show high IFN-γ responses after stimulation with viable IBV. These responses are slightly lower after stimulation with UV-inactivated IBV, especially at 10 dpi. In the second experiment, responses to both IBV and ConA appear to be absent at 10 dpi, whereas some response to UV-inactivated IBV can still be found. No significant differences were found between groups for any of the stimuli. No correlation was found between the IFN-γ production and clinical scores.

4. Discussion

We examined whether immunizations with a modified Semliki Forest Virus-based DNA plasmid expressing the IBV nucleocapsid protein would protect birds against IBV challenge. We looked at clinical signs of IBV infection and examined both the humoral and the cell-mediated immune response following virus challenge.

Expression of the IBV_N protein was confirmed by immunofluorescent labeling of a protein-associated FLAG tag in transfected BHK-21 cells. Two infection experiments were performed in white leghorn chickens ($B^{19}B^{19}$ and $B^{21}B^{21}$), both consisting of two or three immunizations with IBV_N followed by intratracheal and

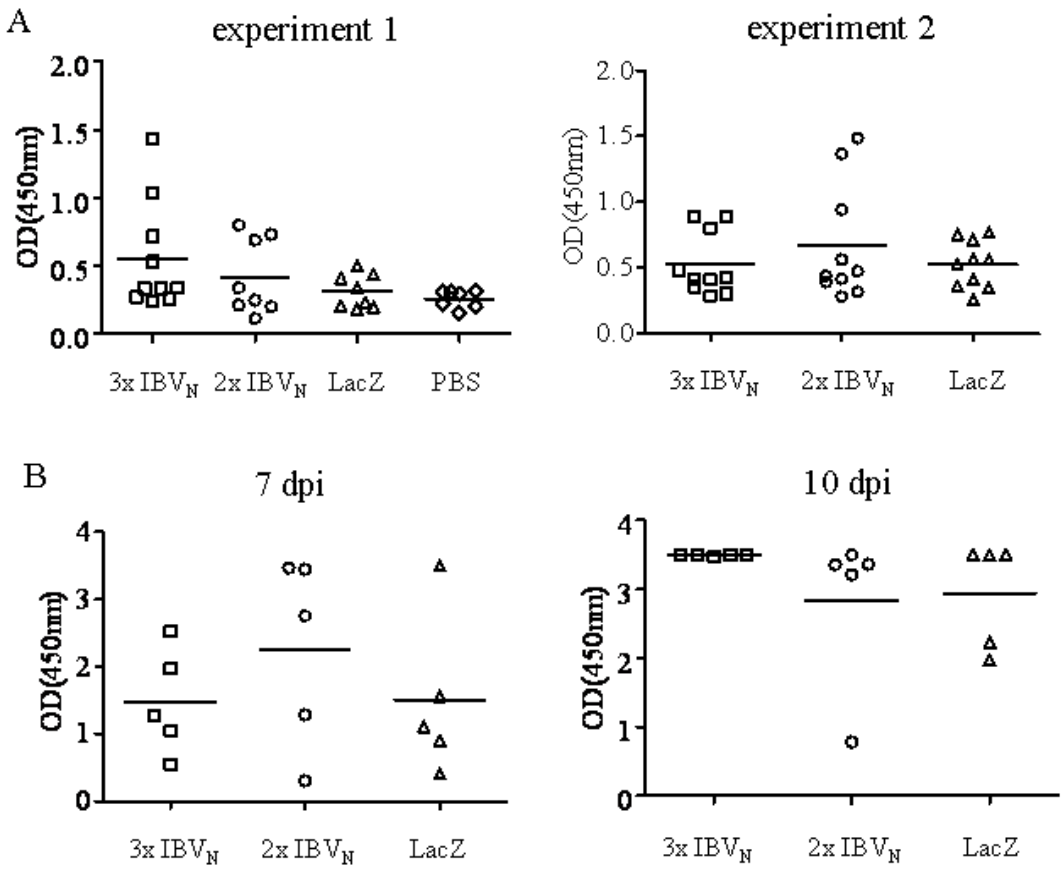

Figure 5. *ELISA to detect antibodies against IBV nucleocapsid protein, after immunization but before IBV infection (A), and 7 and 10 days after IBV infection (B; data are shown for experiment 2 only, data not available for experiment 1). Each dot represents the average OD of three samplings from one chicken.*

eye-nose drop challenge with virulent IBV M41 virus. In the first experiment, chickens were divided into four groups, two of which (PBS and LacZ group) were considered negative control groups. Since no significant difference in their response to IBV challenge was found, we excluded the PBS control group from the second experiment.

Previously, Seo et al. (1997) showed that immunization of chickens with a mammalian expression vector (pTargeT) containing carboxyl-terminal fragments of the IBV_N gene resulted in IBV-specific CTL activity and protection from severe illness. However, they chose injection intervals of no more than 4 days between two consecutive immunizations and the IBV infection, and pretreated the injection area with an anaesthetic agent (bupivacaine) which could have influenced the immune response. In literature (reviewed by Oshop et al., 2002), periods of one to four weeks between DNA immunizations and infection are usually considered most effective in generating an immune response in the avian. We therefore chose to allow intervals of 10 to 12 days between the three immunization rounds and the subsequent virus infection.

An amount of 30 µg of plasmid was chosen per immunization, which is in line with earlier immunization experiments in chickens using the same plasmid (Phenix et al., 2001), although higher doses have also been described (Kapczynski et al., 2003; Seo et al., 1997). The presence of the subgenomic promoter in our construct and the resulting boost to the transcription of the gene of interest should provide

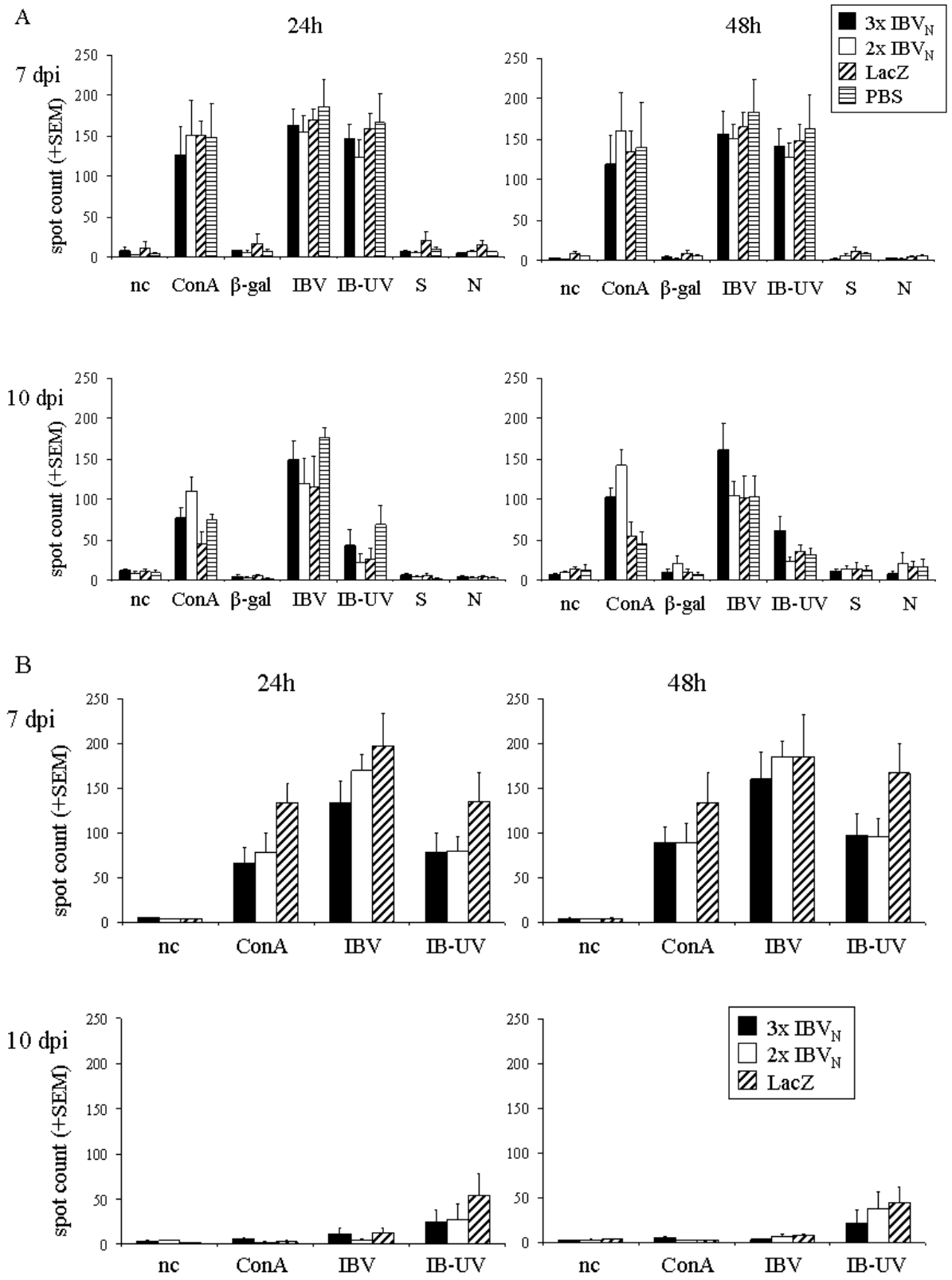

Figure 6. *The number of splenocytes at 7 or 10 days after IBV challenge from experiment 1 (A) and 2 (B) that produced IFN-γ after 24 or 48 h in vitro incubation without any added stimulus (nc) or with the following stimuli: ConA (10 µg/ml), β-galactosidase (10 µg/ml), IBV (10^5 EID_{50}), UV-inactivated IBV (IB-UV; 10^5 EID_{50}), or selected spike (S) or nucleocapsid (N) peptides (10 µg/ml). Bars represent the average spot count per treatment group (+ SEM).*

adequate antigen exposure to the immune system, although the effect of using a higher plasmid dose would be an interesting topic for future studies.

In the first experiment, chickens were divided into four groups, two of which (the PBS control group and the LacZ group) were considered negative control groups. After it was found that the control groups did not differ significantly in their response to IBV challenge, it was decided to exclude the PBS control group from the second experiment.

In both experiments, clinical signs were most apparent in the first two days after infection and decreased gradually between 4 and 6 dpi. Clinical signs were not detected after 7 dpi, which is in line with previous findings (Matthijs et al., 2003). In both experiments the IBV infection was successfully established as confirmed by serological tests. In both experiments moderate to severe clinical signs of IBV infection were found in chickens that received the LacZ plasmid or PBS. In contrast, 10 out of 20 birds in the 3x IBV_N group and 8 out of 18 chickens in the 2x IBV_N group showed only mild signs of infection. This difference was significant ($P<0.05$) between the 2x IBV_N group and the two control groups in the first experiment. Taken together, these experiments support a protective effect of immunization with the IBV_N plasmids.

The immune responses underlying this protective effect were studied using IBV-specific ELISA and IFN-γ ELISPOT assays. No nucleocapsid-specific antibodies were detected in the sera of chickens from the control groups after three immunization rounds prior to IBV infection. In contrast, a third of the birds in each of the IBV_N immunization groups did show an increased antibody response to IBV nucleocapsid protein, but this difference was not significant.

After IBV challenge, we did not find increased humoral responses or a booster effect in the groups that were immunized with IBV_N plasmids. All sera had IBV_N-specific antibodies after IBV infection, with no significant differences in amount or isotype (IgM or IgG; data not shown) found between the treatment groups. The high anti-IBV_N antibody titers found are in line with earlier observations by Ignjatovic and Galli (1993). They found that the IBV_N protein was the immunodominant IBV protein, inducing cross-reactive antibodies with high titers. Immunization with the purified IBV_N protein did not induce protection against challenge with virulent IBV.

To determine whether anti-viral T cell responses were induced by the plasmid immunizations, we examined differences in IFN-γ production by splenocytes in response to *in vitro* restimulation with IBV or β-galactosidase at 7 or 10 dpi, using an ELISPOT assay. No β-galactosidase specific IFN-γ responses were detected in any of the chickens in either experiment. All chickens showed high IFN-γ production after stimulation with IBV, with no significant differences between groups.

In conclusion, we showed a partial protective effect of immunization with the IBV_N plasmid on IBV infection. We also found a slight increase in IBV_N-specific antibodies in birds immunized with the IBV_N plasmid when compared to control birds. Due to the non-antigen specific IFN-γ response, we were not able to evaluate the anti-viral T-cell responses.

References

Albert, M.L., Sauter, B., Bhardwaj, N., 1998, Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 392, 86-89.

Cavanagh, D., 2003, Severe acute respiratory syndrome vaccine development:

experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol. 32, 567-582.
Chen, H., Coote, B., Attree, S., Hiscox, J.A., 2003, Evaluation of a nucleoprotein-based enzyme-linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus. Avian Pathol. 32, 519-526.
Cumberbatch, J.A., Brewer, D., Vidavsky, I., Sharif, S., 2006, Chicken major histocompatibility complex class II molecules of the B19 haplotype present self and foreign peptides. Anim. Genet. 37, 393-396.
DiCiommo, D.P., Bremner, R., 1998, Rapid, high level protein production using DNA-based Semliki Forest virus vectors. J. Biol. Chem. 273, 18060-18066.
Hofmann, A., Plachy, J., Hunt, L., Kaufman, J., Hala, K., 2003, v-src oncogene-specific carboxy-terminal peptide is immunoprotective against Rous sarcoma growth in chickens with MHC class I allele B-F12. Vaccine 21, 4694-4699.
Ignjatovic, J., Galli, L., 1993, Structural proteins of avian infectious bronchitis virus: role in immunity and protection. Adv. Exp. Med. Biol. 342, 449-453.
Ignjatovic, J., Sapats, S., 2000, Avian infectious bronchitis virus. Rev. Sci. Tech. 19, 493-508.
Kapczynski, D.R., Hilt, D.A., Shapiro, D., Sellers, H.S., Jackwood, M.W., 2003, Protection of chickens from infectious bronchitis by in ovo and intramuscular vaccination with a DNA vaccine expressing the S1 glycoprotein. Avian Dis. 47, 272-285.
Lee, S.-Y., Kim, S.H., Kim, V.N., Hwang, J.H., Jin, M., Lee, J., Kim, S., 1999, Heterologous Gene Expression in Avian Cells: Potential as a Producer of Recombinant Proteins. J. Biomed. Sci. 6, 8-17.
Li, J., Huang, Y., Liang, X., Lu, M., Li, L., Yu, L., Deng, R., 2003, Plasmid DNA encoding antigens of infectious bursal disease viruses induce protective immune responses in chickens: factors influencing efficacy. Virus Res. 98, 63-74.
Matthijs, M.G., van Eck, J.H., Landman, W.J., Stegeman, J.A., 2003, Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. Avian Pathol. 32, 473-481.
Miller, D.S., Kotlarski, I., Jilbert, A.R., 2006, DNA vaccines expressing the duck hepatitis B virus surface proteins lead to reduced numbers of infected hepatocytes and protect ducks against the development of chronic infection in a virus dose-dependent manner. Virology 351, 159-169.
Oshop, G.L., Elankumaran, S., Heckert, R.A., 2002, DNA vaccination in the avian. Vet. Immunol. Immunopathol. 89, 1-12.
Pei, J., Sekellick, M.J., Marcus, P.I., Choi, I.S., Collisson, E.W., 2001, Chicken interferon type I inhibits infectious bronchitis virus replication and associated respiratory illness. J. Interf. Cytok. Res. 21, 1071-1077.
Phenix, K.V., Wark, K., Luke, C.J., Skinner, M.A., Smyth, J.A., Mawhinney, K.A., Todd, D., 2001, Recombinant Semliki Forest virus vector exhibits potential for avian virus vaccine development. Vaccine 19, 3116-3123.
Seo, S.H., Collisson, E.W., 1998, Cytotoxic T lymphocyte responses to infectious bronchitis virus infection. Adv. Exp. Med. Biol. 440, 455-460.
Seo, S.H., Wang, L., Smith, R., Collisson, E.W., 1997, The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. J Virol 71, 7889-7894.
Stevenson, M.M., Riley, E.M., 2004, Innate immunity to malaria. Nat. Rev. Immunol. 4, 169-180.

Suarez, D.L., Schultz-Cherry, S., 2000, The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis. 44, 861-868.
Thacker, E.L., Fulton, J.E., Hunt, H.D., 1995, In vitro analysis of a primary, major histocompatibility complex (MHC)-restricted, cytotoxic T-lymphocyte response to avian leukosis virus (ALV), using target cells expressing MHC class I cDNA inserted into a recombinant ALV vector. J. Virol. 69, 6439-6444.
Tischer, B.K., Schumacher, D., Beer, M., Beyer, J., Teifke, J.P., Osterrieder, K., Wink, K., Zelnik, V., Fehler, F., Osterrieder, N., 2002, A DNA vaccine containing an infectious Marek's disease virus genome can confer protection against tumorigenic Marek's disease in chickens. J. Gen. Virol. 83, 2367-2376.
Weinstock, D., Schat, K.A., Calnek, B.W., 1989, Cytotoxic T lymphocytes in reticuloendotheliosis virus-infected chickens. Eur. J. Immunol. 19, 267-272.
Williams, A.K., Wang, L., Sneed, L.W., Collisson, E.W., 1992, Comparative analyses of the nucleocapsid genes of several strains of infectious bronchitis virus and other coronaviruses. Virus Res. 25, 213-222.

Abstract

CHAPTER 5

Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with *E. coli*

Mieke G.R. Matthijs[1], Mark P. Ariaans[2], R. Marius Dwars[1], Jo H.H. van Eck[1], Annemarie Bouma[1], Arjan Stegeman[1], and Lonneke Vervelde[2]

[1]Department of Infectious Diseases and Immunology,
[2]Department of Farm Animal Health,
Faculty of Veterinary Medicine, Utrecht University,
Yalelaan 1, 3584 CL Utrecht, The Netherlands

Veterinary Immunology and Immunopathology, accepted for publication (2008)

Colibacillosis results from infection with avian pathogenic *Escherichia coli* bacteria. Healthy broilers are resistant to inhaled *E. coli*, but previous infection with vaccine or virulent strains of Infectious Bronchitis Virus (IBV) predisposes birds for severe colibacillosis. The aim of this study was to investigate how IBV affects the course of events upon infection with *E. coli*. Broilers were inoculated with IBV H120 vaccine virus or virulent M41 and challenged 5 days later with *E. coli* 506. A PBS and *E. coli* group without previous virus inoculation were included. Sections of trachea, lung and airsacs were stained for CD4, CD8, γδ-TCR, αβ1-TCR, and for macrophages (KUL-01) and both pathogens. Changes in the mucociliary barrier of trachea, lung and airsacs did not predispose for bacterial superinfection. The disease in the lungs of the *E. coli* group and both IBV / *E. coli* groups was similar. Lesions in the airsacs were more pronounced and of longer duration in the IBV / *E. coli* groups. The immunocytological changes differed substantially between the *E. coli* group and both IBV / *E. coli* groups. In trachea, lungs and airsacs the $CD4^+$ and $CD8^+$ populations were significantly larger than in the *E. coli* and PBS groups. In the lungs and the airsacs the macrophages were more numerous in the IBV / *E. coli* and the *E. coli* groups than in the PBS group. The presence of high numbers of T cells and macrophages in IBV infected birds most likely induced an altered immune response, which is responsible for the enhanced clinical signs of colibacillosis.

Key words: chicken, infection, immunology, IBV, vaccine

1. Introduction

In humans as well as in animals it is well known that viral infection of the respiratory tract can predispose for bacterial infections (reviewed by Heinzelmann et al., 2002; Matthijs et al., 2003). Two hypotheses for the underlying mechanisms have emerged. One hypothesis is that bacterial superinfection emerges from viral damage to the respiratory tissue, characterized by loss of cilia and ciliated cells (Bakaletz, 1995), decreased ciliary activity and mucociliary clearance (Wilson et al., 1996), and / or that damage to epithelium may provide more attachment sites to bacteria (El Ahmer et al., 1999). A second hypothesis is that the immune responses after viral infection may increase the susceptibility for bacterial infections.

Phagocytosis of bacteria by macrophages (Debets-Ossenkopp et al., 1982) or neutrophils (Engelich et al., 2001; Navarini et al., 2006) was shown to be hampered due to a previous viral infection. Moreover, the innate anti-viral responses, especially type I IFN, may have a severe granulotoxic effect that increases susceptibility to bacterial superinfection (Navarini et al., 2006). The cell populations in tissues of superinfected animals differ significantly from cell populations in tissues of animals infected with only one pathogen. In addition, superinfections may result in an overproduction of inflammatory cytokines, which may contribute to immunopathology due to exacerbated immune responses (Beadling and Slifka, 2004; Sluijs et al., 2006; Speshock et al., 2007).

Two hypotheses to explain the enhanced susceptibility have been suggested but the underlying mechanisms are still not fully understood (Navarini et al., 2006). The results are not conclusive and further experimental studies are needed to elucidate this phenomenon. Most experiments concerning superinfections of the respiratory tract have been performed in laboratory animals (Beadling and Slifka, 2004; Sluijs et al., 2006). These experimental models have several advantages, but an important disadvantage is that the animals used are usually not the natural host for the infectious agents used.

The use of chickens as experimental animal in combination with avian pathogens could overcome this disadvantage. A superinfection model with Infectious Bronchitis Virus (IBV) and *E. coli* in chickens has been developed (Goren, 1978). Matthijs et al. (2003) used this model and showed increased susceptibility for *E. coli* after previous infection with virulent IBV. Remarkably, this phenomenon was also observed after infection with a mild IBV vaccine virus. This suggested that mechanical tissue damage was not the cause of the enhanced susceptibility to *E. coli*.

This infection model offers the opportunity to investigate how a preceding infection with either a virulent or a mild IBV strain may affect the course of a subsequent *E. coli* infection in the respiratory tract (trachea, lung and airsac) of broilers. The aim of this study was therefore to investigate two possible mechanisms of enhanced susceptibility: tissue damage and alteration of the immune response. The immunocytological changes were observed over a time course from 0.5 hours to 7 days after application of *E. coli* in the trachea. During that period dynamics of IBV, *E. coli*, lymphocytes (CD4, CD8, γδ-TCR and αβ1-TCR) and macrophages (KUL-01) in the trachea, lung and airsacs of broilers were studied.

2. Materials and Methods

2.1 Experimental chickens

Eighteen-day-incubated eggs originating from a *Mycoplasma gallisepticum*-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the department of Farm Animal Health (Utrecht University). From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) each with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 m^3 h^{-1}.

Broilers were fed a commercial ration containing 12.4 MJ of metabolically energy per Kg and 19.5% crude protein *ad libitum*, but from day 14 onwards feed was restricted to 75% of *ad libitum* intake on 'skip a day base' to diminish leg disorders and hydrops ascites. Tap water was provided *ad libitum* throughout the experimental period. Up to the start of feed restriction, 22 h light was given per 24 h; thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35ºC at day 1 to 20ºC at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20ºC.

All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

2.2 Inocula

IBV vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials, which contained at least $10^{3.0}$ EID_{50} (egg infective dose 50%) per dose (Nobilis® IB H120; batch 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, The Netherlands, as freeze-dried vials containing $10^{8.3}$ EID_{50} /1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water just prior to use, and contained at least $10^{3.0}$ EID_{50} /ml of H120 virus and $10^{4.6}$ EID_{50}/ml of IBV M41 virus.

The *Escherichia coli* strain 506 (O78; K80) was isolated from a commercial broiler (Van Eck and Goren, 1991). The *E. coli* culture was prepared as described by Matthijs et al. (2003) and was used at a concentration of $10^{7.6}$ cfu/ml.

2.3 Experimental design

At day 1, broilers were randomly divided into four groups of 30 chickens each. Each group was housed in a separate isolator. At 27 days of age all groups were inoculated oculonasally (one droplet of 0.05 ml per bird in each eye and nostril) and intra-tracheally (1 ml per bird): groups 1 and 2 received distilled water, group 3 received IBV H120 vaccine and group 4 IBV strain M41. At 32 days of age, groups 2, 3 and 4 were intra-tracheally inoculated with 1 ml *E. coli* culture per bird. Group 1 received 1 ml PBS-diluted glucose broth intra-tracheally per bird.

For convenience, we refer to group 1 (distilled water and PBS broth) as PBS group, group 2 (distilled water and *E. coli* broth) as the *E. coli* group, group 3 (IBV H120 vaccine and *E. coli* broth) as the H120 group, and group 4 (IBV strain M41and *E. coli* broth) as M41 group.

2.4 Clinical and post mortem examination

Clinical signs of IBV infection were recorded 1, 2, 4 and 5 days after IBV inoculation and after *E. coli* inoculation and just before euthanizing. A bird was

recorded as having signs of IBV infection if mucous nasal discharge was observed after mild pressure on the nostrils (Matthijs et al., 2003).

From each group, 5 broilers were electrocuted and bled at 0.5 hour, 3 hours, and at days 1, 2, 4 and 7 after *E. coli* inoculation (hpi/dpi). At each time point, colibacillosis lesions were scored macroscopically. Lesion scoring was performed as described by Van Eck and Goren (1991) in the left and right thoracic airsac, pericardium and liver. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird (Van Eck and Goren, 1991).

2.5 Immunocytochemical staining

After post-mortem examination, trachea, left lung and both thoracic airsacs were collected and snap-frozen in liquid nitrogen for immunocytological examination. Samples for immunocytological staining were taken from the middle of trachea, in the area of the entrance of the mediodorsal secondary bronchi in the lung and of both thoracic airsacs in total.

Cryostat sections (6 µm) were transferred to Superfrost Plus slides (Menzel-Glaser) and stored over silicagel for at least 24 h before use. Slides were fixed in pure acetone, air-dried and incubated with mAbs in appropriate concentrations for 1 h; mouse anti-chicken CD4, CD8β, γδ-TCR, αβ1-TCR and KUL-01 (Southern Biotechnology, Birmingham, USA). All mAbs were diluted in PBS containing 1% BSA (Sigma) and 0.1% sodium azide (PBS/BSA).

Slides were washed in PBS and incubated for 1 h with biotinylated horse anti-mouse IgG (Vectastain®). Slides were washed with PBS and incubated for 1 h with avidin:biotinylated enzyme complex (Vectastain® Elite ABC kit) diluted in PBS. Then the slides were incubated with 1 mg 3, 3-diaminobenzidine-tetrahydrochloride (DAB, Sigma, USA) per ml Tris-HCl buffer (0.05 M, pH 7.6) containing 0.06% H_2O_2. To ensure no over- or under-staining, slides were monitored during the reaction under the microscope. Slides were washed with PBS, counterstained with haematoxylin and mounted in Glycergel mounting medium (Dako, USA). All incubations were performed at room temperature in a humidified box. Control slides were incubated as described above, except that mAbs were omitted.

2.6 Immunocytological analysis of the tissue sections

2.6.1 Trachea

Three sections of the trachea per mAbs (CD4, CD8, γδ-TCR, αβ1-TCR and KUL-01) were examined by light microscopy magnification. The score given per animal was the cell count of the 3 trachea rings in total divided by three. Cell populations were scored using the following system for CD4, CD8, KUL-01, γδ-TCR and αβ1-TCR cells: 0 = 0-3 cells, 1 = 4-10 cells, 2 = 11 cells or 1 cluster of cells, 3 = multiple clusters. A cluster is a group of tens of cells located close together.

2.6.2 Left Lung

Of each left lung and for each mAbs (CD4, CD8, γδ-TCR, αβ1-TCR and KUL-01) three parabronchi in the proximity of the Bronchial Associated Lymphoid Tissue (BALT), but not next to the BALT, were analysed. This area was chosen to examine the same well-defined area in every lung consistently. The score given per animal was the total cell count of three parabronchi divided by three. Cell populations were scored using the following system for CD4: 1 = 0-20 cells, 2 = more than 20 cells and / or 1 cluster, 3 = multiple cell clusters; for CD8: 1 = 0-10 cells, 2 = 11-20 cells,

3 = more than 20 cells; for KUL-01: 1 = 20-50 cells, 2 = 51-200 cells, 3 = more than 200 cells; for γδ-TCR and αβ1-TCR: 1 = less than 10 cells, 2 = 11-40 cells, 3 = more than 40 cells.

2.6.3 Left en right thoracic airsacs

Because of lack of a well-defined morphological unit in the airsacs the complete section of the airsacs was examined. Four levels of relative cell density were distinguished for all cell subpopulations: 0 = no cell influx, 1 = few scattered cells throughout the stroma, 2 = many cells scattered throughout the stroma, 3 = complete cell infiltration of the stroma of the airsac wall.

2.7 Detection of IBV and E. coli

2.7.1 Production of polyclonal antibodies against E. coli 506

The bacterial inocula were prepared by submersing one frozen bead (-70°C) of a batch containing the *E. coli* 506 strain in trypton soya broth and by subsequent incubation for 20 h at 37°C. The bacteria were washed with PBS and killed with methanol during 5 min. The solution was centrifuged 10 min. at 1500 x g and the bacteria were washed twice with PBS and dissolved in 10 mg dimethyl dioctadecylammonium bromide (DDA)/ml PBS. Two rabbits were injected 3 times, 21 days between each injections, subcutaneous with 1.5 ml of the PBS/DDA solution containing 10^9 *E. coli* bacteria per ml. Pre-immune and immune sera were tested for specific antibodies against *E. coli* 506 on dot blot, paraffin and cryostat sections. Both live and fixed bacteria were recognized.

2.7.2 Tissue staining procedure for IBV and E. coli

Trachea and lung were collected in 10% buffered formalin, fixed for 24 h and processed to paraffin. Sections were cut 5 µm thick, deparaffinised and placed in pure methanol with 0.75% H_2O_2 for 30 minutes to remove endogenous peroxidase activity.

Sections were incubated with rabbit serum specific for *E. coli* (*E. coli* 506) or with mouse sera with mAbs 48.4 against IBV nucleoprotein (Koch et al., 1990) for 1 h. After washing in PBS, the slides were co-incubated for 1 h with rabbit anti-mouse serum (Dako, Denmark) or goat anti-rabbit serum (Dako, Denmark) conjugated to horseradish peroxidase. The slides were washed and stained with 0.5 mg 3,3-diaminobenzidine-tetrahydrochloride (DAB, Sigma USA) per ml Tris-HCl buffer (0.05 M, pH 7.6) containing 0.03% H_2O_2.

For IBV and *E. coli* staining in the airsac, cryostat sections (6 µm thick) were made from both thoracic airsacs in total. The procedure was the same as used in the immunohistochemical staining. The complete section of each organ was examined for general pathology and the location of both pathogens. The number of *E. coli* bacteria in different parts of the respiratory tract were recorded and classified. Four classes were distinguished: 0, 1, 2, and 3. For trachea and bronchi, the following classes were distinguished: in class 0 no bacteria were found; in class 1 1-5 bacteria; in class 2 6-10, and in class 3 more than 10 bacteria were counted. For the air sacs the classes were: class 0 when no bacteria were found; class 1 for 1-20 bacteria; class 2 20-100 bacteria and class 3 when more than 100 were counted.

2.8 Statistical analysis

Between-group differences per time point were non-parametrically analysed

for mean lesion score (MLS) using the Mann-Whitney test. Nasal discharge, airsacculitis and pericarditis / perihepatitis per time point were non-parametrically analysed between groups with Fischer Exact Test with Bonferroni correction. Lymphocyte subpopulations were analyzed with Fischer Exact Test with the Bonferroni correction.

Examination of birds has not taken place for all organs at the same time points. For the statistical analysis of the airsac all time points were used, but for the other organs the time points of 0.5 and 3 hpi are missing. Both airsacs were examined separately. The classification of birds with respect to the number of *E. coli* was 87% identical; in 6% of the birds a difference of one class was found. Therefore only the right airsac was analysed statistically. Because of the low number of birds per group and time point, no distinction was made between the various classes for number of *E. coli* found. So, for the statistical analysis the result per bird was recorded as 'with (at least one) *E. coli'* or 'no *E. coli*' found (Table 3).

A generalised linear model was performed on the number of birds without or with *E. coli* with a Poisson distribution. The explanatory variables were treatment group (*E. coli*, H120 or M41), time and class (with / without) of *E. coli.* The interaction between time and class and group and class represent the relation between the distributions of the number of birds in the classes with *E. coli*. The best model was based on the (lowest) Akaike's Information Criterion (R development core team, 2007; Pawitan, 2001) but the larger model should have at least a difference of two in AIC to be selected as the best model. For the generalised linear model the statistical program R version 2.5.1 (R Development Core Team, 2007) was used.

3. Results

After inoculation with IBV, the birds were clinically examined, and mucous nasal discharge was observed from 2 to 9 days p.i. (Table 1). After inoculation with *E. coli*, the birds were examined for clinical signs of colibacillosis, and post mortem examination was performed to quantify colibacillosis lesions. The number of birds with airsacculitis, perihepatitis and / or pericarditis and mean lesion score

Table 1. *Number of broilers with mucous nasal discharge at different time points after inoculation with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with* E. coli *at 32 days of age.*

	Days after IBV inoculation							
Group	1	2	4	5*	6	7	9	12
PBS [$]	0/30 [A]	0/30 [A]	0/30 [A]	0/30 [A]	0/5 [A]	0/5 [A]	0/5 [A]	0/5 [A]
E. coli [$$]	0/30 [A]	0/30 [A]	0/30 [A]	0/30 [A]	0/5 [A]	0/5 [A]	0/5 [A]	0/5 [A]
H120	0/30 [A]	1/30 [A]	2/30 [A]	3/30 [A]	1/5 [A]	2/5 [A]	3/5 [A]	0/5 [A]
M41	0/30 [A]	15/30 [B]	28/30 [B]	30/30 [B]	3/5 [A]	3/5 [A]	1/5 [A]	0/5 [A]

[AB] *Groups with different letters within a column are significantly different (P<0.05).*
[$] *Broilers inoculated at 27 d with distilled water and at 32 d with PBS broth.*
[$$] *Broilers inoculated at 27 d with distilled water and at 32 d with* E. coli *broth.*
* *Day of inoculation with* E. coli*.*

Table 2. *Macroscopical colibacillosis lesions: number of broilers with airsacculitis and / or pericarditis / perihepatitis and the mean lesion score (MLS ± SD).*

		Time after *E. coli* 506 strain inoculation					
Broilers with	Group	0.5 h	3 h	1 d	2 d	4 d	7 d
Airsacculitis	PBS [$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	E. coli [$$]	0^{A}	0^{A}	5^{B}	2^{AB}	3^{AB}	0^{A}
	H120	4^{AB}	5^{B}	5^{B}	5^{B}	5^{B}	5^{B}
	M41	5^{B}	5^{B}	5^{B}	4^{AB}	5^{B}	5^{B}
Perihepatitis and / or pericarditis	PBS [$]	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	E. coli [$$]	0^{A}	0^{A}	1^{A}	0^{A}	2^{A}	0^{A}
	H120	0^{A}	0^{A}	0^{A}	0^{A}	2^{A}	1^{A}
	M41	0^{A}	0^{A}	0^{A}	0^{A}	3^{A}	2^{A}
MLS	PBS [$]	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}
	E. coli [$$]	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	1.6 ± 1.6^{B}	1.6 ± 2.2^{AB}	3.4 ± 3.4^{AB}	0.0 ± 0.0^{A}
	H120	0.4 ± 0.2^{A}	0.5 ± 0.0^{A}	2.4 ± 1.5^{B}	3.4 ± 1.9^{B}	6.6 ± 3.5^{B}	5.0 ± 4.1^{B}
	M41	0.5 ± 0.0^{A}	0.5 ± 0.0	3.4 ± 0.9^{B}	2.0 ± 1.9^{B}	6.4 ± 2.5^{B}	4.5 ± 3^{B}

At each time point and within each group five birds were analysed. Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine H120 (group: H120) or virulent IBV M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with E. coli *at 32 days of age.*
[AB] *Groups with different letters within a column are significantly different (P<0.05).*
[$] *Broilers inoculated at 27 d with distilled water and at 32 d with PBS broth.*
[$$] *Broilers inoculated at 27 d with distilled water and at 32 d with* E. coli *broth.*

(macroscopical lesions) are presented in Table 2.

3.1 Trachea

3.1.1 Location of pathogens

H120 virus was detected for 4 days after *E. coli* inoculation in 15 broilers, and M41 virus was detected for 2 days in 6 broilers. Virus was found in the columnar ciliated cells, and in the lamina propria. We did not find any *E. coli* bacteria either attached to the epithelium or in the underlying tissue of the trachea (Table 3). No time effect was found in number of birds with *E. coli*. In birds in the H120 group, *E. coli* was more often found than in birds of the *E. coli* group and the M41 group. No difference was found in number of birds with *E. coli* between the *E. coli* and M41 groups.

3.1.2 Immunocytological changes

In the PBS and the *E. coli* group no influx of lymphocytes was found. The few lymphoid cells found in the lamina propria were $CD4^+$ cells and macrophages. In contrast, in the H120 and the M41 group massive lymphocyte infiltrations were found at all time points after *E. coli* challenge. These infiltrates consisted of $CD4^+$ and $CD8^+$ lymphocytes, and macrophages (Figure 1A). The $CD4^+$ cells expressed the $\alpha\beta1\text{-}TCR^+$, whereas the $CD8^+$ cells expressed either the $\gamma\delta\text{-}TCR^+$ or $\alpha\beta1\text{-}TCR^+$. No significant differences in number of $CD4^+$ and $CD8^+$ cells were found between the H120 and the M41 groups. From 2 dpi onwards the expression of the T cell

Table 3. *Number of birds with or without* E. coli *bacteria in different parts of the respiratory tract detected by immunostaining.*

		Time after *E. coli* infection					
Tissue	Group	0.5h	3h	1d	2d	4d	7d
Right airsac	PBS	0/5 [$]	0/5	0/5	0/5	0/5	0/5
	E. coli	2/3	2/3	3/2	2/3	2/3	0/5
	H120	2/3	1/4	1/4	1/4	0/5	0/5
	M41	1/4	1/4	1/4	1/4	0/5	0/5
Trachea	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	E. coli	n.d.	n.d.	1/4	0/5	0/5	0/5
	H120	n.d.	1/4	1/3	2/2	1/4	0/5
	M41	n.d.	1/4	1/4	0/5	0/4	0/5
Parabronchi	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	E. coli	n.d.	n.d.	5/0	5/0	4/1	2/3
	H120	n.d.	3/2	2/3	4/1	5/0	2/3
	M41	n.d.	4/1	3/1	3/1	2/2	2/3
Bronchi	PBS	n.d.	n.d.	0/5	0/5	0/5	0/5
	E. coli	n.d.	n.d.	5/0	4/1	1/3	0/5
	H120	n.d.	4/1	1/3	2/2	2/3	0/5
	M41	n.d.	3/2	2/2	0/5	1/3	0/4

Birds were inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age. All birds except those in the PBS group were inoculated with E. coli *at 32 days of age. At each time point and within each group five birds were analysed.*
[$] 0/5 : the number of birds with at least one E. coli detected / the number of birds without E. coli *in that part of the respiratory tract.*
n.d. = not determined

receptors, both γδ-TCR and αβ1-TCR, reduced drastically. The expression of CD4 and CD8 in tissues of birds in these groups remained high (data not shown).

All cell subpopulations were found scattered throughout the tissue, but $CD4^+$ and $CD8^+$ cells were also found in clusters. The $CD8^+$ cells were found around B cell follicles, and $CD4^+$ cells were located within B cell follicles.

3.2 Lung

3.2.1 Location of pathogens

IBV (H120 and M41) was found in the columnar ciliated cells of the epithelium in the bronchi, but not in the parabronchus. *E. coli* was found as free bacteria and agglomerates in the capillary area, atria, and pus within parabronchial and bronchial lumina (Table 3). At 2 dpi and 4 dpi *E. coli* was mainly found as small agglomerates in the capillary part and lumen of the parabronchus. The presence of bacteria was accompanied by inflammatory changes of the parabronchi (data not shown). In both the parabronchi and secondary bronchi no time or group effect was found in the number of broilers with *E. coli*.

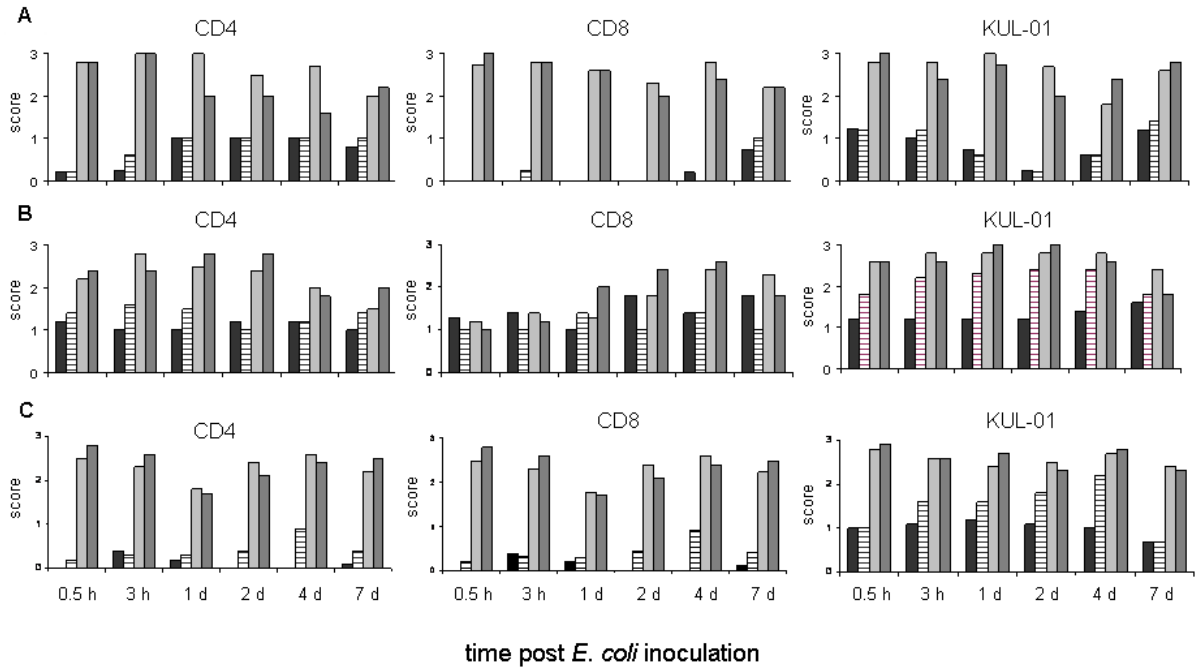

Figure 1. *Number of CD4$^+$, CD8$^+$ and KUL-01$^+$ cells in different parts of the respiratory tract of broilers inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41). All birds except those in the PBS group were inoculated with* E. coli *at 5 dpi. At each time point and within each group five birds were analysed. Black bars: PBS group; hatched bars:* E. coli *group; grey bars: H120 group; dark grey bars: M41 group.*
A) <u>trachea:</u> mean cell count per three trachea rings. CD4$^+$, CD8$^+$, KUL-01$^+$ cells: 0: 0-3 cells, 1: 4-10 cells, 2: 11 cells or 1 cluster of cells, 3: more than 1 cell cluster. A cluster is a group of tens of cells located close together.
B) <u>lungs:</u> mean cell count per three parabronchi. CD4$^+$ cells: 1: 0-20 cells, 2: more than 20 cells and 1 cluster, 3: multiple clusters; CD8$^+$ cells: 1: 0-10 cells, 2: 11-20 cells, 3: more than 20 cells; KUL-01: 1: 20-50, 2: 51-200, 3: more than 200 cells.
C) <u>airsacs:</u> the complete section of the airsac was examined. CD4, CD8, KUL-01: 0 = no cell influx, 1 = few scattered cells throughout stroma, 2 = many cells scattered throughout stroma, 3 = massive cell infiltration of stroma of airsac wall.

3.2.2 Immunocytological changes

The number of affected parabronchi and the severity of affection of parabronchi are presented in Table 4. At 0.5 hpi many CD4$^+$ cells and macrophages were present in lungs of IBV infected birds. At that time no difference in number of CD8$^+$ cells was found between the different groups (Figure 1B). From 1 to 7 d after *E. coli* infection, an increase in CD8$^+$ cells was detected in the M41 group and at 4 and 7 dpi in the H120 group. The numbers of CD4$^+$, CD8$^+$ and KUL-01$^+$ cells in H120 and M41 groups significantly exceeded the numbers of these cells in the *E. coli* group.

In H120 and M41 groups many macrophages were present until 4 dpi. In the *E. coli* group, the number of macrophages increased after inoculation, but did not equal the levels in the H120 and M41 groups. At 7 dpi the number of macrophages was decreased in the *E. coli* and M41 groups to the level found in the PBS group, but it remained high in the H120 group.

Up to 2 dpi most macrophages were elongated, whereas from 4 dpi most macrophages were enlarged and rounded up suggesting activation of these cells.

Table 4. *Percentage of damaged parabronchi in broilers inoculated with IBV vaccine H120 (group: H120) or virulent IBV strain M41 (group: M41) at 27 days of age.*

	Damaged parabronchi					Severity of damage				
	Time after *E. coli* inoculation									
Group	3 h	1 d	2 d	4 d	7 d	3 h	1 d	2 d	4 d	7 d
PBS [$]	n.d.[$$$]	-	-	-	-	n.d.	-	-	-	-
E. coli [$$]	n.d.	++	++	+	+/-	n.d.	++	+	+/-	+/-
H120	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-
M41	+	++	+	+/-	+/-	+/-	+	+/-	+/-	+/-

All birds except those in the PBS group were inoculated with E. coli *at 32 days of age. At each time point and within each group five birds were analysed.*
Percentage of damaged parabronchi per field section: -: 0%, +/-: 0-10%, +: 10-30%, ++: more than 30%. Severity of damage of the parabronchi: -: no changes, +/-: minor cell infiltration/oedema in part of parabronchus, +: generalized cell infiltration in whole parabronchus, ++: massive cell infiltration and/or necrosis in parabronchus.
[$] *Broilers inoculated at 27 d with distilled water and at 32 d with PBS broth.*
[$$] *Broilers inoculated at 27 d with distilled water and at 32 d with* E. coli *broth.*
[$$$] *n.d. = not determined.*

No significant differences in the number of γδ-TCR$^+$ cells in the lung were found between the four groups. In the H120 and M41 groups, the number of αβ1-TCR$^+$ cells increased 4 dpi coinciding with the increase of CD8$^+$ cells (TCR data not shown; Figure 1B). No significant difference between H120 and M41 groups were found in numbers of CD4$^+$, CD8$^+$, KUL-01$^+$, γδ-TCR$^+$ and αβ1-TCR$^+$ cells.

3.3 Airsac

3.3.1 Location of pathogens

IBV was found in the columnar ciliated cells of respiratory epithelium in the airsacs of birds in the H120 and M41 groups; H120 virus was detected for 2 days after *E. coli* inoculation in 14 birds, and M41 virus was detected for 3 hours after *E. coli* inoculation in 9 birds. *E. coli* was found predominantly in pus located at the respiratory surface in the airsacs. For the right airsac no group effect was found on number of birds with *E. coli* (Table 3).

3.3.2 Immunocytological changes

In the PBS and *E. coli* groups only few CD4$^+$ cells were observed. In contrast, massive infiltrations of CD4$^+$ and CD8$^+$ lymphocytes were found in birds in the H120 and M41 groups from 0.5 hpi until 7 dpi (Figure 1C). Macrophages were present in H120 and M41 groups at 0.5 hpi, and their number remained high up to 7 dpi (Figure 1C). In the PBS and *E. coli* groups, few macrophages were present at 0.5 hpi. Only in the *E. coli* group a substantial increase was found which was highest at 4 dpi. Nevertheless, the number of macrophages in this group remained lower than the number of macrophages in the H120 and M41 groups.

At 7 dpi the number of macrophages in the *E. coli* group decreased to a level comparable to the level of the birds in the PBS group (Figure 1C). The numbers of CD4$^+$, CD8$^+$ and KUL-01$^+$ cells in H120 and M41 groups exceeded significantly the

numbers of these cells in the *E. coli* group. In the H120 and M41 groups the number of γδ-TCR$^+$ cells and αβ1-TCR$^+$ cells was high from 0.5 hpi till the end of the experimental period. In the *E. coli* group an increase of γδ-TCR$^+$ cells occurred from 0.5 hpi to 4 dpi occurred, which was decreased at 7 dpi. Few γδ-TCR$^+$ cells were found in the PBS group and hardly any αβ1-TCR$^+$ cells were found in the PBS and *E. coli* groups. No significant differences in number of CD4$^+$, CD8$^+$, KUL-01$^+$, γδ-TCR$^+$ and αβ1-TCR$^+$ cells were detected between the H120 and M41 groups.

4. Discussion

In this study the course of an *E. coli* infection after a previous infection with a virulent (M41) or vaccine (H120) strain of IBV in trachea, lungs and airsacs of broilers was examined. The aim of this study was to investigate two possible mechanisms of enhanced susceptibility: tissue damage and alteration of the immune response.

Despite significant differences in number of birds with nasal discharge 2 to 5 days after inoculation with either IBV strain, comparable clinical, macroscopical and microscopical changes were observed after infection with *E. coli* in both groups. Remarkably, 4 dpi mean lesion scores were comparable in both the *E. coli* and IBV groups, whereas at 7 dpi birds in the *E. coli* group were fully recovered but birds in both IBV groups still showed signs of colibacillosis. These findings suggest that due to a previous infection with either IBV strain birds were less capable of conquering the damage of the *E. coli* infection.

In the trachea, epithelial damage and mononuclear cell infiltration due to infection with IBV was found, however no additional effect of the subsequent infection with *E. coli* was noticed, indicating that the apparent changes did not result in predisposition of the trachea for bacterial superinfection. In the lungs of both the *E. coli* group and the IBV groups acute purulent pneumonia of similar severity was observed, suggesting no additional effect of IBV on acute pneumonia. Moreover, the course of the disease in the lungs in both the *E. coli* group and the IBV groups was similar and birds of these groups were nearly completely recovered from pneumonia at 7 dpi. Although apoptosis of granulocytes and subsequent leukopenia is described after viral infection (McCullers, 2006; Navarini et al., 2006) the massive granulocyte infiltration in the lungs and the presence of pus in the airsacs in both the *E. coli* and the IBV / *E. coli* infected birds did not clearly suggest a reduction in the participation of granulocytes in the process. These findings suggest that neither trachea nor lungs (as suggested by DeRosa et al., 1992; Smith et al., 1985) were predilection sites for bacterial superinfection.

Lesions in the airsacs of both IBV groups were more pronounced and of longer duration than in the *E. coli* group. In contrast to the lungs, the airsacs from IBV infected birds were not recovered at 7 dpi whereas the airsacs of only *E. coli* infected birds were. The airsacs were the only location where both pathogens were found at the same time. Our results indicate that the difference in reaction upon superinfection with *E. coli* in IBV infected broilers compared to an *E. coli* infection without preceding virus infection is not caused by damage to the mucociliary barrier facilitating bacterial adhesion or penetration or by reduced clearance of bacteria.

The second hypothesis was that alteration of the immune response by a virus infection could make birds more susceptible to subsequent bacterial infections. At time of infection with *E. coli*, great numbers of CD4 cells, CD8 cells and macrophages were found in trachea and airsacs of birds of the IBV groups, whereas

great numbers of CD4 cells and macrophages were found in the lung. The pronounced presence of macrophages at time of challenge with *E. coli* most likely increased the clearance of the bacteria, since fewer bacteria were found in lungs and airsacs of IBV infected groups. This finding suggests a reduced susceptibility for a superinfection with *E. coli*. However, macrophages produce type I IFNs (IFN-α/β) after viral infection which have potent effects on viral replication, including that of IBV, but these cytokines can inhibit host defence against both Gram-positive and Gram-negative bacteria such as *Listeria monocytogenes* (O'Connell et al., 2004), *Mycobacterium tuberculosis* in the lungs (Manca et al., 1002), and *Salmonella typhimurium* (Navarini et al., 2006), and possibly also against *E. coli* in broilers. The CD4 cells and CD8 cells also present in large numbers in IBV groups can produce type II IFN (IFN-γ) which is known to have a protective role in both viral and bacterial infections through the induction of a Th1 cell immune response (Decker et al., 2002). However, its role in the activation of macrophages and airway epithelial cells can lead to an exaggerated inflammatory response (Konno et al., 2002). Both T cells and macrophages present at time of *E. coli* inoculation might therefore be responsible for the enhanced colibacillosis, due to overproduction of inflammatory cytokines.

In the airsacs, the numbers of macrophages remained high in the IBV groups, whereas the number of macrophages in the *E. coli* group decreased at 7 dpi to the level of the PBS group. Although both IBV and *E. coli* were cleared in all groups, the macrophages were still present in the airsacs at 7 dpi in the IBV groups. It is unclear whether the macrophages and T cells in the trachea, lungs and airsacs are functionally altered by the IBV infection, but the clear difference in the presence of these cells in IBV infected birds both at time of challenge and in the airsacs at 7 dpi might suggests that an altered microenvironment resulted in altered immune responses, as for example demonstrated in mice where lymphocytes were detrimental during the early innate immune responses against *Listeria monocytogenes* due to increased apoptosis inhibiting effector reactions (Carrero et al., 2006). Both T cells and macrophages present at the time of *E. coli* inoculation might therefore be responsible for the increase of both the severity and the duration of the inflammatory reactions found in the airsacs in both IBV / *E. coli* infected groups, due to overproduction of inflammatory cytokines, as demonstrated in a study with turkeys (Rautenschlein et al., 1998).

Ariaans et al. (2008) found that the phagocytic capacity and NO production of peripheral blood mononuclear cells and splenocytes was not affected by prior exposure to IBV, but that the proinflammatory response in the spleen of IBV infected birds seemed severely impaired compared to only *E. coli* infected birds, and suggested that the virus modulated the innate immunity of the birds. Our study only showed immunocytological changes, as no functional study was performed.

In this study, only a limited number of samples were examined. However, the samples were considered representative, because they were taken in a uniform and reproducible manner and the lesions within each compartment were distributed evenly.

It is concluded that a preceding infection of the respiratory tract with IBV does not predispose for bacterial superinfection with *E. coli* by altering the mucociliary barrier, but likely moderates the immune response.

Acknowledgements

The authors thank J.C.M. Vernooij for performing the statistical analysis.

References

Ariaans, M.P., Matthijs, M.G., Van Haarlem, D., Van de Haar, P.M., Van Eck, J.H., Hensen, E.J., Vervelde, L., 2008. The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after Infectious Bronchitis Virus infection. Vet. Immunol. Immunopathol. 123: 240-250.

Bakaletz, L.O., 1995. Viral potentiation of bacterial superinfection of the respiratory tract. Trends Microbiol. 3, 110-114.

Beadling, C., Slifka, M.K., 2004. How do viral infections predispose patients to bacterial infections? Curr. Opin. Infect. Dis. 17, 185-191.

Carrero, J.A., Calderon, B. Unanue, E.R., 2006. Lymphocytes are detrimental during the early innate immune response against *Listeria monocytogenes*. J. Exp. Med. 203, 933-940.

Debets-Ossenkopp, Y., Mills, E.L., Van Dijk, W.C., Verbrugh, H.A., Verhoef., J., 1982. Effect of influenza virus on phagocytic cells. Eur. J. Clin. Microbiol. 1, 171-177.

Decker T., Stockinger, S., Karaghiosoff, M., Müller, M., Kovarik, P., 2002. IFNs and STATs in innate immunity to microorganisms. J. Clin. Invest. 109, 1271-1277.

DeRosa M., Ficken, M.D., Barnes, H.J., 1992. Acute airsacculitis in untreated and cyclophosphamide-pretreated broiler chickens inoculated with *Escherichia coli* or *Escherichia coli* cell-free culture filtrate. Vet. Pathol. 29, 68-78.

El Ahmer, O.R., Raza, M.W., Ogilvie, M.M., Weir, D.M., Blackwell, C.C., 1999. Binding of bacteria to HEp-2 cells infected with influenza A virus. FEMS Immunol. Med. Mic. 23, 331-341.

Engelich, G., White, M., Hartshorn, K.L., 2001. Role of the respiratory burst in co-operative reduction in neutrophil survival by influenza A virus and *Escherichia coli*. J. Leukocyte Biol. 69, 50-56.

Goren, E. 1978. Observations on experimental infection of chicks with *Escherichia coli.* Avian Pathol. 7, 213-224.

Heinzelmann, M., Scott, M., Lam, T., 2002. Factors predisposing to bacterial invasion and infection. Am. J. Surg. 183, 179-190.

Koch, G., Hartog, L., Kant, A., K.L. Van Roozelaar, D.J., 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. J. Gen. Virol. 71, 1929-1935.

Konno, S., Grindle, K.A., Lee, W.M., Schroth, M.K., Mosser, A.G., Brockman-Schneider, R.A., Busse, W.W., Gern, J.E., 2002. Interferon-γ enhances rhinovirus-induced RANTES secretion by airway epithelial cells. Am. J. Resp. Cell. Mol. 26, 594-601.

Manca, C., Tsenova, L., Bergtold, A., Freeman, S., Tovey, M., Musser, J.M., Barry, C.E., Freedman, V.H., Kaplan, G., 2001. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α/β. P. Natl. Acad. Sci. USA. 98, 5752-5757.

Matthijs, M.G., Van Eck, J.H., Landman, W.J., Stegeman, J.A., 2003. Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. Avian Pathol. 32, 473-481.

McCullers, J.A. 2006. Insights into the interaction between influenza virus and pneumococcus. Clin. Microbiol. Rev. 19, 571-82.
Navarini, A.A., Recher, M., Lang, K.S., Georgiev, P., Meury, S., Bergthaler, A., Flatz, L., Bille, J., Landmann, r., Odermatt, B., Hengartner, H., Zinkernagel, R.M., 2006. Increased susceptibility to bacterial superinfection as a consequence of innate antiviral responses. P. Natl. Acad. Sci. USA. 103, 15535-15539.
O'Connell, R.M., Saha, S.K., Vaidya, S.A., Bruhn, K.W., Miranda, G.A., Zarnegar, B., Perry, A.K., Nguyen, B.O., Lane, T.F., Taniguchi, T., Miller, J.F., Cheng, G., 2004. Type I interferon production enhances susceptibility to *Listeria monocytogenes* infection. J. Exp. Med. 200, 437-445.
Pawitan, Y., 2001. In all Likelihood: Statistical Modelling and Inference Using Likelihood, ISBN: 019850765, pp 375-382.
R Development Core Team, 2007. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.
Rautenschlein, S., Miller, R.L., Sharma, J.M., 1998. Interferon induction in turkeys by oral administration of the imidazoquinolinamine S-28828 and modulation of the pathogenesis of *Escherichia coli*. Vet. Immunol. Immunopathol. 66, 127-41.
Smith, H.W., Cook, J.K. Parsell, Z.E., 1985. The experimental infection of chickens with mixtures of infectious bronchitis virus and *Escherichia coli*. J. Gen. Virol. 66, 777-786.
Speshock, J.L., Doyon-Reale, N., Rabah, R., Neely, M.N., Roberts, P., 2007. Filamentous Influenza A virus infection predisposes mice to fatal septicaemia following superinfection with *Streptococcus pneumoniae* serotype 3. Infect. Immun. 75, 3102-3111.
Van der Sluijs, K.F., Nijhuis, M., Levels, J.H.M., Florquin, S., Mellor, A.L., Jansen, H.M., Van der Poll, T., Lutter, R., 2006. Influenza-induced expression of indoleamine 2,3-dioxygenase enhances interleukin-10 production and bacterial outgrowth during
secondary pneumococcal pneumonia. J. Infect. Dis. 193, 214-22.
Van Eck, J.H.H., Goren, E., 1991. An Ulster 2C strain-derived Newcastle disease vaccine: vaccinal reaction in comparison with other lentogenic Newcastle disease vaccines. Avian pathol. 20:497-507.
Wilson, R., Dowling, R.B., Jackson, A.D., 1996. The biology of bacterial colonization and invasion of the respiratory mucosa. Eur. Respir. J. 9:1523-1530.

Abstract

CHAPTER 6

The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after Infectious Bronchitis Virus infection

Mark Ariaans[1], Mieke Matthijs[2], Daphne van Haarlem[1], Peter M. van de Haar[1], Jo van Eck[2], Evert Hensen[1], Lonneke Vervelde[1]

[1]Department of Infectious Diseases and Immunology,
[2]Department of Farm Animal Health,
Faculty of Veterinary Medicine, Utrecht University,
Yalelaan 1, 3584 CL Utrecht, The Netherlands

Veterinary Immunology and Immunopathology, 2008; 123: 240-250

Colibacillosis results from infection with avian pathogenic *Escherichia coli* bacteria. Healthy broilers are resistant to inhaled *E. coli*, but previous infection with vaccine or virulent strains of Infectious Bronchitis Virus (IBV) predisposes birds for severe colibacillosis. We investigated whether IBV affects recruitment and function of phagocytic cells and examined NO production, phagocytic and bactericidal activity, and kinetics of peripheral blood mononuclear cells (PBMC) and splenocytes. Moreover, we measured cytokine mRNA expression in lung and spleen samples. Broilers were inoculated with IBV H120 vaccine or virulent M41 and challenged 5 days later with *E. coli* 506. A PBS control and *E. coli* group without previous virus inoculation were also included. Birds were sacrificed at various time points after inoculation (h/dpi). Inoculation with IBV induced extended and more severe colibacillosis than with *E. coli* alone. At 4 dpi, the number of PBMC in all *E. coli*-inoculated groups was significantly higher than in PBS-inoculated birds, which correlated with lesion scores. From 1 to 4 dpi, NO production by PBMC from all *E. coli*-inoculated animals was elevated compared to PBS birds. Bactericidal activity of PBMC in IBV-inoculated animals at 7 dpi was lower than in PBS- and *E. coli*-inoculated birds, but phagocytic capacity and recruitment were not severely impaired. In spleen samples of IBV-infected animals reduced expression of IL-1β, IL-6, IL-8, IL-10, IL-18 and IFN-γ mRNA was found 1 dpi. Our results suggest that enhanced colibacillosis after IBV infection or vaccination is caused at least by altered innate immunity and less by impairment of phagocytic cell function.

Key words: IBV vaccine; colibacillosis; phagocytosis; bactericidal activity; cytokines; innate immunity.

1. Introduction

Predisposition for bacterial infections in the course of respiratory viral infections is found in various species, both avian and mammalian. In chickens, turkeys and ducks colibacillosis is often observed secondary to infection with respiratory agents e.g. Infectious Bronchitis Virus (IBV), Newcastle Disease Virus, and *Mycoplasma gallisepticum* (Igbokwe et al., 1996; Nakamura et al., 1994). In mice, influenza virus increases susceptibility to *Streptococcus pneumoniae* infection (Seki et al., 2004; Speshock et al., 2007) and measles virus predisposes for a range of bacterial infections such as *Listeria monocytogenes* (Slifka et al., 2003). In cattle, susceptibility for pneumonic pasteurellosis is greatly enhanced by a variety of respiratory viruses including bovine respiratory syncytial virus (Liu et al., 1999), bovine herpes virus-1 (Leite et al., 2002) and bovine coronavirus (Storz et al., 2000) . In man, pneumonia has been a leading cause of death during influenza pandemics, supporting the widely held view that influenza virus predisposes for streptococcal infections (McCullers, 2006).

The mechanisms behind enhanced susceptibility to bacterial superinfection after viral infection have been studied extensively (Beadling and Slifka, 2004; Hament et al., 1999) but are still not well understood. A first set of hypotheses suggests increased susceptibility due to tissue damage in the respiratory tract resulting in functional impairment. Three possible causes have been described as mechanisms for functional damage. Viral replication in the upper respiratory tract causes loss of cilia and ciliated cells (Bakaletz, 1995), decreased ciliary activity impairs mucociliary clearance (Wilson et al., 1996) and finally, damage to epithelium may provide more attachment sites for bacteria (Ahmer et al., 1999).

A second set of hypotheses suggests altered innate immune responses. Impairment of innate effector functions, i.e. adhesion and entry, phagocytosis, killing, nitric oxide (NO) and superoxide production have been suggested previously (Ficken et al., 1987; Naqi et al., 2001; Read et al., 1999) . Changes in recruitment or function of macrophages and neutrophils after bacterial superinfections have been described in mice (Navarini et al., 2006; Slifka et al., 2003).

Modulation of other innate functions affecting induction and / or control of adaptive responses might have a long term effect upon the host-pathogen interaction. Type I interferons (IFNs I) induced after viral infection have an antiviral effect, but can be detrimental for induction of anti-bacterial responses (Navarini et al., 2006; O'Connell et al., 2004), whereas type II IFNs play an important role in bacterial infections (Shtrichman et al., 2001).

Goren (1978) developed an experimental model in broilers that demonstrated enhanced susceptibility to *Escherichia coli* after inoculation with IBV. Moreover, not only a virulent strain (IBV M41) enhanced inflammatory reactions on superinfection with *E. coli*, but the mild IBV vaccine strain (IBV H120) widely used in the field also enhanced susceptibility in a very similar way (Matthijs et al., 2003).

In our study to investigate which mechanisms underlie enhanced susceptibility to colibacillosis after IBV infection or vaccination, we examined the dynamics of pathogens and of immunopathological changes (Matthijs et al., accepted 2008 Vet. Immunol. Immunopathol.).

The aim of this study was to detect whether innate effector functions such as the recruitment of effector cells and function of phagocytic cells, measured by NO production and bactericidal capacity, had been affected after IBV infection. As an alternative explanation for the enhanced colibacillosis we also examined altered expression of cytokine mRNA in spleen and lung samples.

2. Materials and methods

2.1. Experimental chickens

Eighteen-day-incubated eggs originating from a *Mycoplasma gallisepticum*-free broiler parent (Cobb) flock were obtained from a commercial hatchery and hatched at the research facility of the department of Farm Animal Health (Utrecht University). From day of hatch (day 1), broilers were housed in negative pressure HEPA isolators (Beyer and Eggelaar, Utrecht, The Netherlands) each with a volume of 0.68 m^3 and fitted with a wire floor of 0.93 m^2. Isolators were ventilated at a rate of approximately 40 m^3 h^{-1}. Broilers were fed a commercial ration containing 12.4 MJ of metabolically energy per Kg and 19.5% crude protein *ad libitum*, but from day 14 onwards feed was restricted to 75% of *ad libitum* intake to diminish leg disorders and hydrops ascites. Tap water was provided *ad libitum* throughout the experimental period. Up to the start of feed restriction, 22 h light was given per 24 h; thereafter it was reduced to 16 h. From day 4 onwards red light was given to prevent cannibalism. Isolator temperature was gradually decreased from 35ºC at day 1 to 20ºC at day 31. From day 31 to the end of the experiment isolator temperature was kept at approximately 20ºC. All chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands, in accordance with the Dutch regulation on experimental animals.

2.2. Inocula

The IB vaccine virus H120 was obtained as commercial freeze-dried 1000 doses vials which contained at least $10^{3.0}$ EID_{50} (egg infective dose 50%) per dose (Nobilis® IB H120; batch 041106A). The virulent Massachusetts IBV strain M41 was obtained from Intervet, Boxmeer, the Netherlands, as freeze-dried vials containing $10^{8.3}$ EID_{50}/1.2 ml/vial. The virus had been passaged twice in SPF embryonated eggs. All IBV inocula were prepared in distilled water just prior to use and contained at least $10^{3.0}$ EID_{50}/ml of H120 virus and $10^{4.6}$ EID_{50}/ml of M41 virus. The *Escherichia coli* strain 506 (O78; K80) was isolated from a commercial broiler (Eck and Goren, 1991). The *E. coli* culture was prepared as described by Matthijs et al. (2003) and was used at a concentration of $10^{7.6}$ cfu/ml.

2.3. Experimental design

At day 1, broilers were randomly divided into four groups of 30 chickens each. Each group was housed in a separate isolator. At 27 days of age, all groups were inoculated oculo-nasally (one droplet of 0.05 ml per bird in each eye and nostril) and intratracheally (1 ml per bird): groups 1 and 2 received distilled water, group 3 received IBV H120 virus and group 4 IBV M41 virus. At 32 days of age, groups 2, 3 and 4 were intratracheally inoculated with 1 ml *E. coli* 506 culture per bird. Group 1 received 1 ml PBS-diluted glucose broth intratracheally per bird. For convenience, we refer to group 1 (distilled water and PBS broth) as PBS group, group 2 (distilled water and *E. coli* broth) as the *E. coli* group, group 3 (IBV H120 virus and *E. coli* broth) as the H120 group, and group 4 (IBV M41 virus and *E. coli* broth) as M41 group.

2.4. Clinical and post-mortem examination

Clinical signs of IBV infection were determined 1, 2, 4 and 5 days after IBV

inoculation and after *E. coli* inoculation just before euthanizing. A bird was recorded as having signs of IBV infection if mucous nasal discharge was observed after mild pressure on the nostrils (Matthijs et al., 2003).

From each group, 5 broilers were electrocuted and blood collected at 0.5 hour, 3 hours and at days 1, 2, 4 and 7 after *E. coli* inoculation (hpi/dpi). At each time point, colibacillosis lesions were scored macroscopically. Lesion scoring was performed as described by Van Eck and Goren (1991) in the left and right thoracic air sac, pericardium and liver. Lesion scores ranged from 0 to 3 per organ, leading to a maximum score of 12 per bird (Matthijs et al., 2005).

2.5. Flow cytometric analysis of cell composition

Peripheral blood, spleen and lung were collected from sacrificed birds. Spleen tissue was squeezed through a 70 µm mesh to prepare single cell suspensions. Splenocytes and PBMC were isolated by density gradient centrifugation for 20 min at 850 x g using Ficoll-Hypaque (density 1.078), washed twice with PBS (Cambrex) and adjusted to $5x10^7$ cells/ml in RPMI1640 medium supplemented with 10% FBS, 2 mM glutamax-I (Gibco) and 100 U/ml penicillin and streptomycin (Gibco).

Cell suspensions were fluorescently labeled for 30 min on ice with mAb against thrombocytes (mouse anti-chicken CD41/CD61:FITC, Serotec), monocytes (mouse anti-chicken monocyte/macrophage KUL-01:FITC, Southern Biotechnology

Table 1. *Real-time quantitative RT-PCR primers and probes.*

RNA target		Probe/primer sequence (5' – 3')	Accession Number
28S	probe	(FAM) - AGGACCGCTACGGACCTCCACCA - (TAMRA)	X59733
	F primer	GGCGAAGCCAGAGGAAACT	
	R primer	GACGACCGATTTGCACGTC	
IL-1β	probe	(FAM) - CCACACTGCAGCTGGAGGAAGCC - (TAMRA)	AJ245728
	F primer	GCTCTACATGTCGTGTGTGATGAG	
	R primer	TGTCGATGTCCCGCATGA	
IL-4	probe	(FAM) - AGCAGCACCTCCCTCAAGGCACC - (TAMRA)	AJ621735
	F primer	AACATGCGTCAGCTCCTGAAT	
	R primer	TCTGCTAGGAACTTCTCCATTGAA	
IL-6	probe	(FAM) - AGGAGAAATGCCTGACGAAGCTCTCCA - (TAMRA)	AJ309540
	F primer	GCTCGCCGGCTTCGA	
	R primer	GGTAGGTCTGAAAGGCGAACAG	
IL-8	probe	(FAM) - TCTTTACCAGCGTCCTACCTTGCGACA - (TAMRA)	AJ009800
	F primer	GCCCTCCTCCTGGTTTCAG	
	R primer	TGGCACCGCAGCTCATT	
IL-10	probe	(FAM) - CGACGATGCGGCGCTGTCA - (TAMRA)	AJ621614
	F primer	CATGCTGCTGGGCCTGAA	
	R primer	CGTCTCCTTGATCTGCTTGATG	
IL-18	probe	(FAM) - CCGCGCCTTCAGCAGGGATG - (TAMRA)	AJ276026
	F primer	AGGTGAAATCTGGCAGTGGAAT	
	R primer	ACCTGGACGCTGAATGCAA	
IFN-α	probe	(FAM) - CTCAACCGGATCCACCGCTACACC - (TAMRA)	U07868
	F primer	GACAGCCAACGCCAAAGC	
	R primer	GTCGCTGCTGTCCAAGCATT	
IFN-β	probe	(FAM) - TTAGCAGCCCACACACTCCAAAACACTG - (TAMRA)	X92479
	F primer	CCTCCAACACCTCTTCAACATG	
	R primer	TGGCGTGCGGTCAAT	
IFN-γ	probe	(FAM) - TGGCCAAGCTCCCGATGAACGA - (TAMRA)	Y07922
	F primer	GTGAAGAAGGTGAAAGATATCATGGA	
	R primer	GCTTTGCGCTGGATTCTCA	

Associates) and $CD8^+$ cells (mouse anti-chicken CD8α:FITC, mouse anti-chicken CD8β:RPE, Southern Biotechnology Associates). Subsequently, cell-associated fluorescence was analysed by flow cytometry using Cell Quest software (Becton Dickinson). Results are expressed as percentages of total viable cells.

2.6. Nitric oxide production assay

Cells were seeded in flatbottom 96-well plates at $2.5x10^6$ cells in 50 ml culture medium (RPMI1640 + 2% chicken serum + 2 mM glutamax-I (Gibco) and 100 U/ml penicillin and streptomycin (Gibco)). Cells were incubated for 48 h at 41°C, 5% CO_2 with 100 ml of either culture medium to determine background production of nitric oxide (NO), medium supplemented with 10 mg/ml heat-killed *E. coli* 506, or medium with 10 mg/ml *E. coli* LPS (from *E. coli* O55:B5, Sigma) to measure maximum NO production. After incubation, 50 ml/well of culture supernatant was transferred to sterile flatbottom 96-well plates. An NO dilution series (200–3.13 mM) was included on each plate as a standard curve to determine the amount of NO produced. To each well, 50 ml of Griess reagent (1% sulfanilamide and 0.3% naphtylenediamine, 1:1 in 2.5% phosphoric acid) was added, plates were incubated for 10 min at room temperature on a plate shaker and absorbance was recorded at 550 nm. For each chicken, NO secretion was calculated as total amount of NO produced by the cells after stimulation with heat-killed *E. coli* or *E. coli* LPS, minus the background NO production by unstimulated cells.

2.7. Phagocytosis and killing assay

Splenocytes and PBMC were incubated for 30 minutes at 10^8 cells in 1 ml RPMI 1640, 5% FBS containing 10^9 *E. coli* bacteria, in roundbottom 12 ml polypropylene tubes (Greiner Bio-One) at 37°C, 5% CO_2, to allow bacterial adhesion and entrance. At this point, gentamycin (200 mg/ml; Gibco) was added to each tube to kill extracellular bacteria and cells were incubated for an additional 30 min at 37° C. Cells were washed once with PBS to remove the gentamycin, and either lysed directly with 1% saponin (T = 0.5 h) to release intracellular bacteria, or incubated for 14 h or 24 h in RPMI 1640 medium supplemented with 5% FBS and 20 mg/ml gentamycin before cell lysis. The cells were cultured in the presence of gentamycin to prevent re-infection and unlimited growth in the medium of *E. coli* released from dead cells. After saponin treatment, cell lysates were centrifuged and pellets were resuspended in 200 ml PBS and plated in duplicate (100 ml lysate per plate) on McConky Agar (MCA) plates (Biotrading) to determine the number of viable bacteria. The MCA plates were incubated overnight at 37°C and colonies were counted blindly. Results were calculated as the sum of colonies on the duplicate plates. If the sum of colony counts on the duplicate plates was below 100 colonies, cells were considered successful in clearing the *E. coli*, whereas cells were considered unable to clear the bacteria when counts were over 300 colonies.

2.8. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA samples isolated from spleen and lung were screened for mRNA encoding IL-1β, IL-4, IL-6, IL-8, IL-10, IL-18, IFN-α, IFN-β, and IFN-γ. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Purified RNA was eluted in 30 µl RNase-free water and stored at -80° C. Reverse transcription was performed using iScript cDNA Synthesis Kit (Biorad). Primers and probes (Applied Biosystems) were designed according to Kaiser et al.

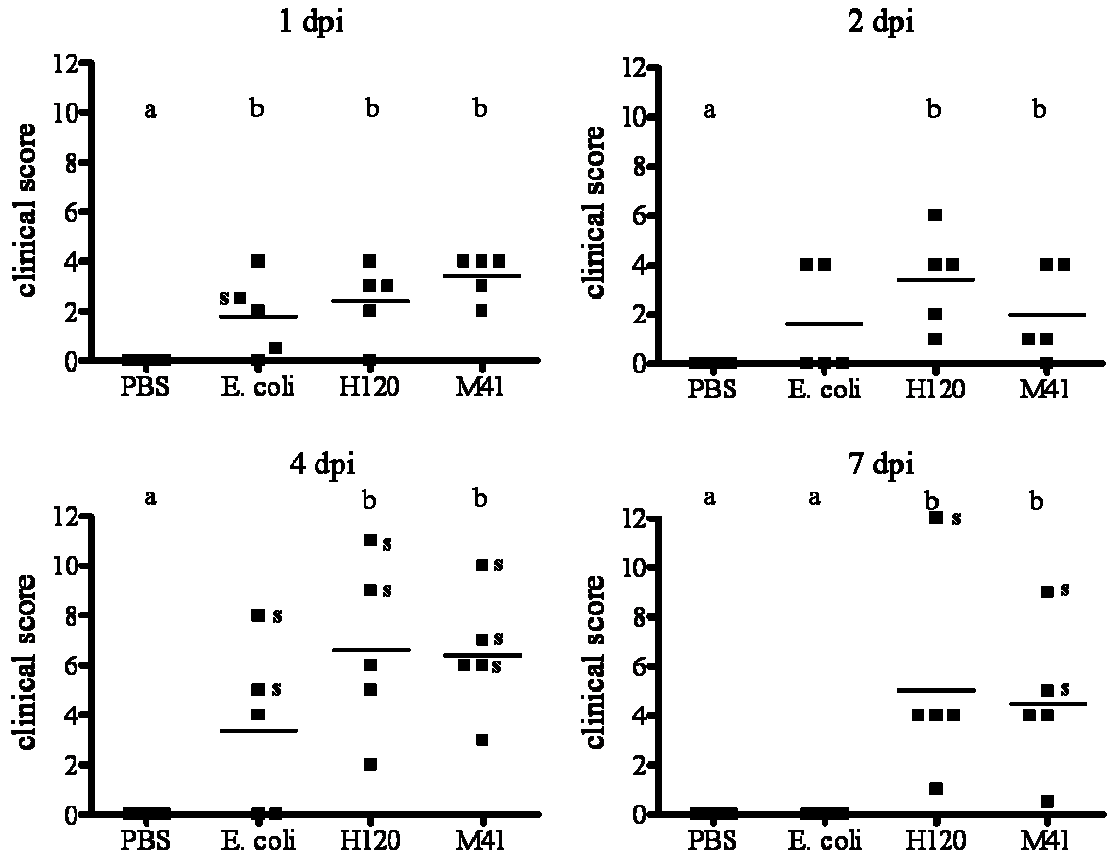

Figure 1. *Colibacillosis lesion scores at different time points after* E. coli *inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before* E. coli *inoculation, whereas the PBS group received no IBV or* E. coli*. Per treatment group, 5 birds were sampled at each time point. Each dot represents the total lesion score of an individual bird with a maximum score of 12. The horizontal lines indicate the mean scores of each treatment group. Birds with systemic signs of colibacillosis, characterized by lesions in liver and/ or pericardium, are designated with 's'. Groups with different letters are significantly different (P<0.05).*

(2003) and Rothwell et al. (2004) and are listed in Table 1. For the quantitative RT-PCR assay TaqMan Universal PCR Master Mix (Applied Biosystems) was used. Primers were used at 300 nM and probes at 100 nM concentration. Amplification and detection of specific products was achieved with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) with the following cycle profile: one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, and 40 cycles of 95°C for 10 sec and 59°C for 1 min. Results were expressed as fold changes between samples, relative to the PBS group (Philbin et al., 2005). To correct for variation in RNA preparation and sampling, Ct values for cytokine-specific product for each sample were standardized using the Ct value of 28S-specific product for the same sample. To normalize RNA levels between samples within an experiment, the mean Ct value for 28S-specific product was calculated by pooling Ct values of all samples in that experiment. Well-to-well variations in Ct value of 28S-specific product about the experimental mean were calculated. The slope of the 28S dilution series regression lines was used to calculate differences in input of total RNA. Fold differences in cytokine expression between treatment groups were calculated relative to the reference ribosomal RNA, using a method adapted from Philbin et al. (2005). Fold

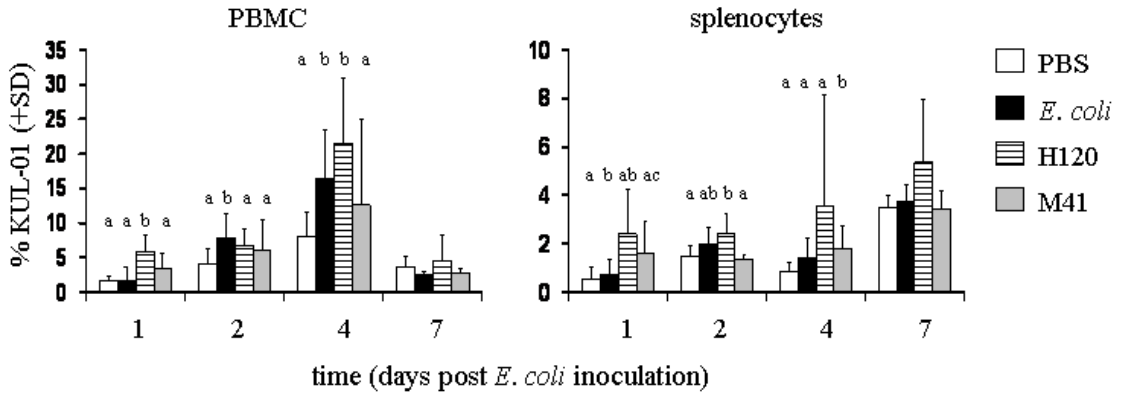

Figure 2. *Percentage (+ SD) of monocytes / macrophages (KUL-01) in PBMC and splenocytes at different time points after* E. coli *inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before* E. coli *inoculation, whereas the PBS group received no IBV or* E. coli*. Bars represent the average frequency of labelled cells per treatment group (n=5), as a percentage of total live cells. Groups with different letters are significantly different (P<0.05).*

differences were calculated from the Ct values C (for the cytokine) and C' (for ribosomal RNA) using the equation $^{10}\log R_{(A/B)} = [(C_A - C_B)/S] - [(C'_A - C'_B)/S']$, where S and S' are, respectively, the slopes of plots of Ct value against the ^{10}logarithm of concentration for serial dilutions of cytokine DNA and ribosomal RNA, assayed on the same plate. This calculation avoids assumptions about the efficiency of the PCR amplifications and reduces to the common ΔΔCt method in the case that both have perfect efficiency.

2.9. Statistical analysis

Between-group differences per time point were non-parametrically analyzed for each assay using the Mann-Whitney test. Partial correlations between different assays were determined per time point, with treatment as a control variable. Analysis was performed using the SPSS program and the probability level for significance was taken as $P < 0.05$.

3. Results

3.1. Clinical and macroscopical observations

Five days after IBV inoculation, all broilers in the M41 group and 10% of the broilers in the H120 group showed nasal discharge. Birds were examined macroscopical for colibacillosis lesions (figure 1). No lesions were observed in birds of the *E. coli* group at 0.5 h and 3 h after *E. coli* inoculation, at these time points all broilers of the H120 and the M41 groups, except one in the H120 group, had airsacculitis. Airsacculitis (colibacillosis lesions in the thoracic airsacs) was observed in chickens of all *E. coli*-inoculated groups between 1 and 4 dpi. At 7 dpi, no signs of colibacillosis were observed anymore in the *E. coli* group whereas all birds of the H120 and the M41 groups had airsaccullitis. Systemic lesions (perihepatitis / pericarditis) were observed in 1 bird of the H120 group and 2 birds of the M41 groups at 7 dpi. No macroscopical signs of colibacillosis were observed in birds of the PBS group throughout the course of the experiment. More detailed clinical data can be found in Matthijs et al. (accepted for publication Vet. Immunol. Immunopathol. 2008).

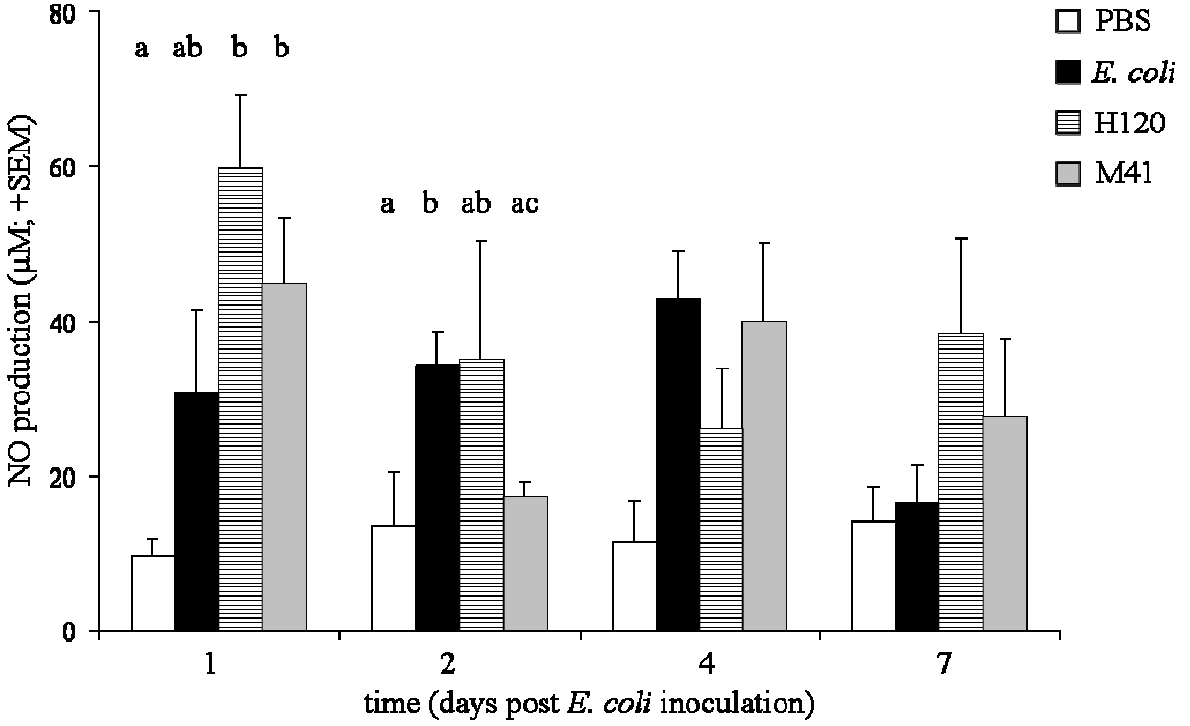

Figure 3. *Means ± SEM of* E. coli*-induced NO production in response to heat-killed* E. coli *506 by PBMC per group, at various time points after* E. coli *inoculation. Groups H120 and M41 were inoculated with their respective IBV strains 5 days before* E. coli *inoculation, whereas the PBS group received no IBV or* E. coli*. NO production by cells of individual chickens is calculated as the total NO production after stimulation with heat-killed* E. coli *or LPS, minus the background NO production of unstimulated cells. Groups with different letters are significantly different (P<0.05).*

3.2. Flow cytometric analysis of blood mononuclear cells and splenocytes

The frequencies of thrombocytes, monocytes / macrophages, $CD8\beta^+$ and $CD8\alpha^+$ cells were analyzed as percentages of total viable cells in PBMC and splenocyte cell suspensions by flow cytometry at 1, 2, 4 and 7 dpi. Thrombocytes accounted for 60-75% of PBMC and 2-4% of splenocytes, with no significant differences between the treatment groups or fluctuations in time (data not shown). The percentage monocytes in peripheral blood of all *E. coli*-inoculated birds increased up to 4 dpi, and at 7 dpi, dropped to levels found in the PBS group (figure 2). A significant positive correlation ($P < 0.01$) between monocyte percentages and colibacillosis lesion scores was found at 4 dpi. Whereas an effect of the *E. coli* inoculation on monocyte frequencies was apparent, monocyte frequencies were not significantly decreased nor increased in IBV-inoculated birds when compared to birds that received *E. coli* only. In the spleen at 1 dpi, a significantly higher percentage of macrophages was found in IBV-infected birds, and only in the H120 group, a higher percentage of macrophages was found at all time points. These changes were not significantly correlated to lesion scores. Significant variations in the percentages of $CD8\beta^+$, PBMC and splenocytes between the treatment groups were observed at different time points, but these variations were not related to an effect of either *E. coli* or IBV (data not shown). Similar fluctuations in the percentages of $CD8\alpha^+\beta^-$ cells were found, apart from 1 dpi, where the M41 group

Table 2. *Phagocytosis and killing of bacteria by PBMC of chickens killed 4 and 7 days after* E. coli *inoculation (dpi).*

Group	colony counts 4 dpi [a]			colony counts 7 dpi [a]		
	< 100	100-300	> 300	< 100	100-300	> 300
PBS [b]	3	0	2	0	4	1
E. coli	0	3	2	2	2	1
H120	0	2	3	1	1	3
M41	1	1	3	0	1	4

Birds were inoculated with Infectious Bronchitis Virus (IBV) vaccine strain H120 (group: H120), virulent IBV strain M41 (group: M41) or PBS (groups: PBS and E. coli*) and 5 days later inoculated with* E. coli*, the PBS group with PBS. Each group consisted of five broilers. Numbers represent the number of chickens in each group displaying the specified killing efficiency. For each chicken, samples were run in duplicate.*

[a] *The number of colonies retrieved from phagocytic cells after lysis was used as a measurement for killing efficiency; retrieval of less than 100 colonies was considered successful clearance, whereas retrieval of more than 300 colonies was considered as a failure to clear* E. coli*.*

[b] *Inoculated at 32 days with glucose broth instead of* E. coli *broth.*

showed higher percentages of $CD8\alpha^{+}\beta^{-}$ cells than the other groups, but no significant correlation was found with the *E. coli* or IBV inoculation (data not shown).

3.3. Nitric oxide production

The Griess reaction assay was used to determine whether IBV inoculation had an effect on the production of NO by splenocytes and PBMC after subsequent stimulation with heat-killed *E. coli* bacteria or *E. coli* LPS. NO production by PBMC of the *E. coli*-inoculated groups at 1 dpi was significantly higher than of the PBS group (figure 3). Furthermore the IBV-inoculated groups, most notably the H120 group, showed higher NO production than the *E. coli* group, but at 2 dpi, NO production of the IBV-inoculated groups almost halved. At 7 dpi, NO production of the *E. coli* group had dropped to the level of the PBS group, whereas NO production by both the H120 and M41 groups was elevated compared to the PBS and *E. coli* groups.

NO production by PBMC and splenocytes after *E. coli* LPS-stimulation showed a pattern comparable to stimulation with heat-killed *E. coli*, although the amount of NO produced was approximately twice as high (data not shown).

3.4. Phagocytosis and killing assay

The ability of PBMC and splenocytes to kill *E. coli* bacteria after internalization was determined at 4 and 7 dpi, based on the number of colonies found after 24 h incubation. Uptake of bacteria was confirmed after 14 h incubation as the number of *E. coli* colonies retrieved from the PBMC and splenocytes and was higher than 300 in all chickens (data not shown). At 7 dpi, in the H120 and M41 groups the PBMC of 3 respectively 4 out of 5 chickens did not kill engulfed bacteria based on high colony counts, whereas only 1 chicken in the PBS group and 1 chicken in the *E. coli* group did not kill intracellular bacteria (table 2). At 4 dpi, splenocytes from all groups successfully killed *E. coli* bacteria. At 7 dpi however, splenocytes from 2 chickens in the H120 group were not able to kill the bacteria

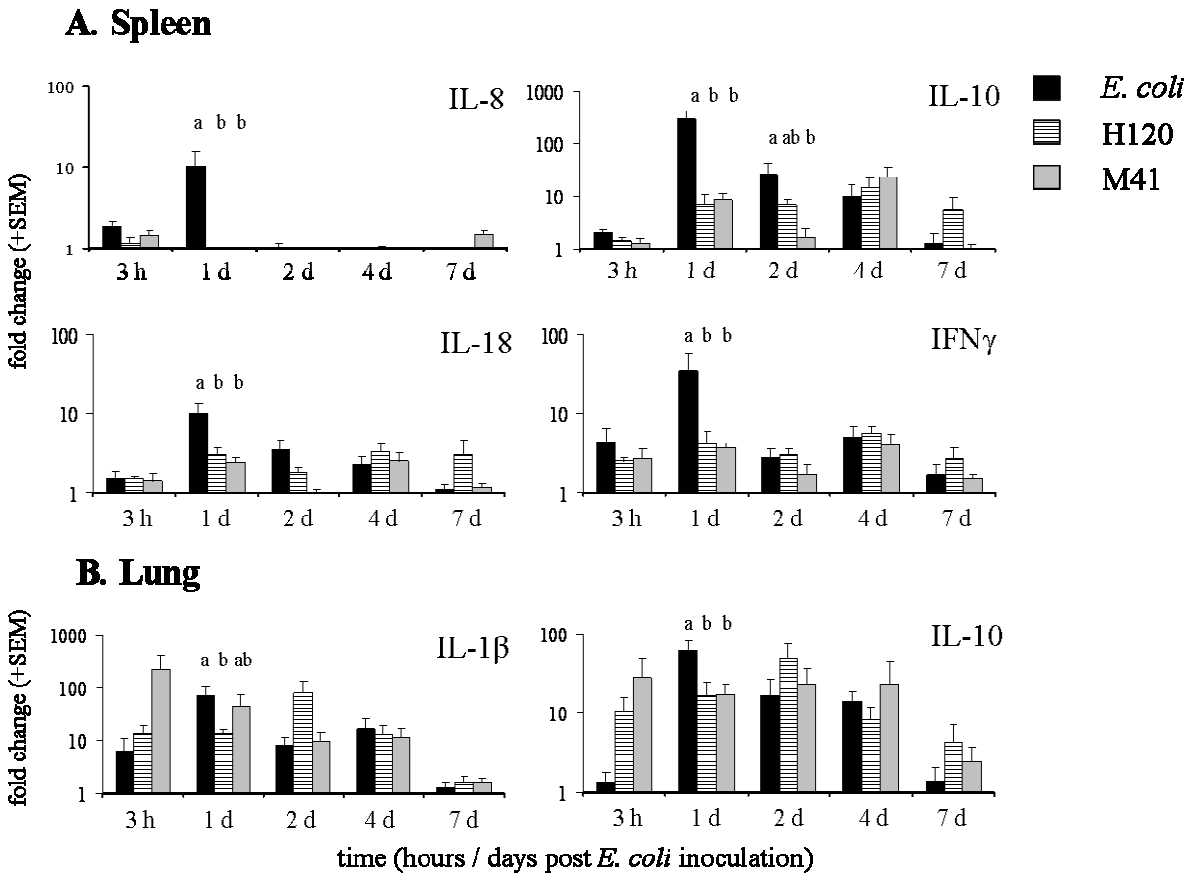

Figure 4. *Real-time quantification of cytokine mRNA expression by cells isolated from A) splenocytes and B) lung tissue samples of PBS chickens (white bars), chickens inoculated with* E. coli *only (black bars), IBV H120 +* E. coli *(hatched bars) and IBV M41 +* E. coli *(grey bars). Data are expressed as mean relative fold increase at different time points after* E. coli *inoculation, compared to samples of PBS birds. Error bars show SEM for triplicate samples of five birds per treatment group. Groups with different letters are significantly different (P<0.05).*

within 24 h incubation (data not shown).

3.5. Real-time quantitative RT-PCR

To examine the effects of IBV and *E. coli* inoculation on systemic immune functions, cytokine mRNA expression of spleen and lung samples were measured by real-time quantitative RT-PCR. No significant differences between the groups were found at any time point for IL-4, IFN-α and IFN-β in either spleen or lung samples (data not shown). At 1 dpi, spleen mRNA levels for IFN-γ and the pro-inflammatory cytokines IL-1β and IL-6 (data not shown) as well as IL-8, IL-18 and IFN-γ (figure 4A) were significantly upregulated in the *E. coli*-inoculated group in comparison to the IBV-inoculated groups. Similarly, mRNA levels for the anti-inflammatory cytokine IL-10 was increased significantly in *E. coli*-inoculated birds compared to the IBV-inoculated birds. The mRNA levels of these cytokines dropped sharply from 2 dpi onward. Interestingly, at 7 dpi, only in the H120 group, IL-6, IL-10 and IFN-γ mRNA expression was still higher than the *E. coli* group. Tests were carried out to find whether altered expression of cytokine mRNA in IBV-inoculated birds was also found in lung samples (figure 4B). In lung samples, no significant differences between the groups were found at any time point for IL-18, IFN-β and

IFN-γ (data not shown). In contrast to our findings in the spleen, at 3 hpi, mRNA levels for IL-6, IL-8, IFN-γ (data not shown), IL-10 and IL-1β (figure 4B) in lung samples of birds of the H120 and especially the M41groups were higher than those of the *E. coli* group. However, at 1 dpi, mRNA expression of these cytokines in the *E. coli* group had increased similar to, or higher than that of the birds in the IBV infected groups. Levels remained elevated at 2 dpi, with highest expression in the IBV H120 group. At 7 dpi, mRNA expression levels of all three *E. coli*-inoculated groups had dropped to the level of the birds of the PBS group.

4. Discussion

Enhanced susceptibility for bacterial infections as a consequence of viral infection is reported in varying combinations in various species (Hament et al., 1999). In poultry, severe colibacillosis after infections with Infectious Bronchitis Virus is well documented (Peighambari et al., 2000; Vandekerchove et al., 2004) and is a serious problem in commercial chickens.

Interestingly, not only the virulent IBV M41 field strain induces enhanced susceptibility to subsequent *E. coli* infection, but the mild H120 vaccine strain also induces this enhanced colibacillosis to about the same level (Matthijs et al., 2003). A model based on these findings was used to study the phenomenon and test the contribution of different possible factors causing the phenomenon. In a separate paper, we addressed the question whether local changes at the surfaces of the trachea, lung and airsacs might facilitate local bacterial growth and subsequent entry of bacteria. The results of that study showed that the clearance of the bacteria from the lung was comparable for all the groups inoculated with *E. coli*, irrespective of previous exposure to virus (Matthijs et al., accepted Vet. Immunol. Immunopathol. 2008).

In this paper we examined whether prior exposure to IBV virus (M41 or H120) affected the effector functions of mononuclear cells resulting in the phenomenon of enhanced susceptibility, a hypothesis supported by several papers (Ficken et al., 1987; Naqi et al., 2001; Slifka et al., 2003). For this question, NO production by mononuclear cells isolated from the spleen and PBMC were tested. After *E. coli* inoculation, the NO production in samples upon stimulation with heat-killed *E. coli* bacteria or *E. coli* LPS (data not shown) was strongly increased in all groups, irrespective of prior exposure to IBV, although at 1 dpi the increase was highest in IBV-inoculated birds. Another difference was observed at 7 dpi, where birds exposed to *E. coli* alone showed diminished NO production compared to birds also exposed to either IBV strain (M41 or H120). This is in line with the clinical picture of bacterial persistence in broilers previously exposed to IBV.

Subsequently, the internalization and intracellular killing of bacteria was tested in splenocytes and PBMC of all groups. Again all groups exposed to *E. coli*, irrespective of earlier exposure to IBV, responded similarly in the assay at 4 dpi, in that the cells had a diminished killing capacity. At a later time point (7 dpi), bactericidal capacity in the groups exposed to IBV was still lower than the bactericidal capacity of the group exposed to *E. coli* alone, suggesting a slower recovery. This once more was in line with the clinical recovery observed in the *E. coli* alone group.

The results of the NO production and of the internalization and intracellular killing of bacteria by the individual splenocytes or PBMCs suggest that the small changes in effector functions of those cells cannot be the only reason for the

phenomenon of enhanced colibacillosis in animals previously exposed to virus. However, changes in the number of cells and / or their recruitment could still influence the outcome of the secondary infection. We therefore evaluated the percentage of effector cells by FACS analysis. Major differences that could explain the enhanced colibacillosis in the samples of the dual infected groups were not found.

Because no major changes in the effector functions of the mononuclear cells could be found to explain the phenomenon of enhanced colibacillosis, other mechanisms to explain the findings were sought. Supported by the observation that clinical colibacillosis seemed mainly the result of a systemic effect, and not directly of a local lung condition, we set out to find other explanations and looked at whether cytokine profiles would clarify the clinical picture. This might lead to insights into how the innate capacity of the animals was modulated at the time of *E. coli* exposure. Therefore, we examined mRNA expression in splenocytes and in samples of the lung.

After *E. coli* inoculation alone, the pro-inflammatory cytokines IL-1β and IL-6 were strongly increased in both splenocytes and lung tissue. This was expected, because both cytokines play an important role in initiating an acute-phase immune response against invading pathogens and activating a wide range of immune cells such as macrophages and T cells (Wigley and Kaiser, 2003).

The pro-inflammatory chemokine IL-8 was found to be upregulated in lung samples as early as 3 hpi, and in splenocytes at 1 dpi. The findings in the lung are in agreement with its function as a chemotractant, produced at the infection site in order to recruit heterophils and initiate a rapid local inflammatory response (Withanage et al., 2004). The upregulation in spleen samples was only found in the *E. coli* group and is likely due to the fact that the bacteria are not retained in the respiratory tract but spread systemically. Surprisingly, in the IBV groups no IL-8 mRNA is found in the spleen, which might suggest that a lack of response in the spleen can contribute to prolonged colibacillosis. The mRNA expression of pro-inflammatory mediators IL-1β and IL-6 in the spleen at 1 dpi in IBV groups also lagged behind that of the *E. coli* group.

Not only the cytokines IFN-γ and IL-18, but also IL-10 were found to be upregulated in splenocytes and lung samples of birds inoculated with *E.coli* alone. IFN-γ is a potent macrophage-activating factor and IL-18 is an important inducer of IFN-γ production (Wigley and Kaiser, 2003). Both IFN-γ and IL-18 are strongly linked to a cell-mediated Th1-like immune response (Staeheli et al., 2001). IL-10 on the other hand is a promotor of Th2-like immune responses, predominantly by inhibiting pro-inflammatory and Th1 cytokines (Rothwell et al., 2004), which can increase host susceptibility to bacterial diseases through its anti-inflammatory effects, including suppression of macrophage function. Previous studies in chickens showed that IL-10 production could be accompanied by either downregulation of IFN-γ production (Abdul-Careem et al., 2007; Rothwell et al., 2004), but also by concurrent upregulation of IL-10 and IFN-γ (Hong et al., 2006). Moreover, it should be noted that IL-10 is known to have immunostimulatory effects in certain species, inducing MHC class II upregulation and cytotoxic T cell stimulation (Groux and Cottrez, 2003).

In our chicken model, we find that high mRNA expression of pro- and anti-inflammatory cytokines at 1 dpi in spleen samples is not found in the birds that were inoculated with IBV and *E. coli*, but only in the *E. coli* inoculated birds, and thus seems to be associated with recovery of colibacillosis. The differences in mRNA

expression are consistent with the finding that virus-induced modulation of the immune response plays an important role in the susceptibility to subsequent bacterial infection.

In conclusion, we observed that both vaccine and virulent IBV caused enhanced colibacillosis, but IBV did not significantly affect phagocytic capacity and NO production of peripheral mononuclear cells and splenocytes. However, IBV did alter the systemic cytokine mRNA expression patterns after *E. coli* inoculation in commercial broilers likely resulting in enhanced colibacillosis.

Acknowledgements

The authors gratefully thank S. Reemers for technical assistance in this study and dr. A. Bouma for criticically reading the manuscript.

References

Abdul-Careem, M.F., Hunter, B.D., Parvizi, P., Haghighi, H.R., Thanthrige-Don, N., Sharif, S., 2007, Cytokine gene expression patterns associated with immunization against Marek's disease in chickens. Vaccine 25, 424-432.

Ahmer, O.R., Raza, M.W., Ogilvie, M.M., Weir, D.M., Blackwell, C.C., 1999, Binding of bacteria to HEp-2 cells infected with influenza A virus. FEMS Immunol. Med. Mic. 23, 331-341.

Bakaletz, L.O., 1995, Viral potentiation of bacterial superinfection of the respiratory tract. Trends Microbiol. 3, 110-114.

Beadling, C., Slifka, M.K., 2004, How do viral infections predispose patients to bacterial infections? Curr. Opin. Infect. Dis. 17, 185-191.

Eck, J.H.H.v., Goren, E., 1991, An Ulster 2C strain-derived Newcastle disease vaccine: vaccinal reaction in comparison with other lentogenic Newcastle disease vaccines. Avian Pathol. 20, 497-507.

Ficken, M.D., Edwards, J.F., Lay, J.C., 1987, Effects of Newcastle disease virus infection on the binding, phagocytic, and bactericidal activities of respiratory macrophages of the turkey. Avian Dis. 31, 888-894.

Goren, E., 1978, Observations on experimental infection of chicks with *Escherichia coli*. Avian Pathol. 7, 213-224.

Groux, H., Cottrez, F., 2003, The complex role of interleukin-10 in autoimmunity. J. Autoimmun. 20, 281-285.

Hament, J.M., Kimpen, J.L., Fleer, A., Wolfs, T.F., 1999, Respiratory viral infection predisposing for bacterial disease: a concise review. FEMS Immunol. Med. Microbiol. 26, 189-195.

Hong, Y.H., Lillehoj, H.S., Lee, S.H., Dalloul, R.A., Lillehoj, E.P., 2006, Analysis of chicken cytokine and chemokine gene expression following Eimeria acervulina and Eimeria tenella infections. Vet. Immunol. Immunopathol. 114, 209-223.

Igbokwe, I.O., Salako, M.A., Rabo, J.S., Hassan, S.U., 1996, Outbreak of infectious bursal disease associated with acute septicaemic colibacillosis in adult prelayer hens. Rev. Elev. Med. Vet. Pays Trop. 49, 110-113.

Kaiser, P., Underwood, G., Davison, F., 2003, Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. J. Virol. 77, 762-768.

Leite, F., Sylte, M.J., rsquo, Brien, S., Schultz, R., Peek, S., van Reeth, K., Czuprynski, C.J., 2002, Effect of experimental infection of cattle with bovine herpesvirus-1 (BHV-1) on the ex vivo interaction of bovine leukocytes with Mannheimia (Pasteurella) haemolytica leukotoxin. Vet. Immunol. Immunopathol. 84, 97-110.

Liu, L., Lehmkuhl, H.D., Kaeberle, M.L., 1999, Synergistic effects of bovine respiratory syncytial virus and non-cytopathic bovine viral diarrhea virus infection on selected bovine alveolar macrophage functions. Can. J. Vet. Res. 63, 41-48.

Matthijs, M.G., Ariaans, M.P., R.M. Dwars, J.H.H. van Eck, Bouma, A., Stegeman, A., Vervelde, L., 2008, Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with E. coli. Accepted for publication in Vet. Immunol. Immunopathol.

Matthijs, M.G., van Eck, J.H., de Wit, J.J., Bouma, A., Stegeman, J.A., 2005, Effect of IBV-H120 vaccination in broilers on colibacillosis susceptibility after infection with a virulent Massachusetts-type IBV strain. Avian Dis. 49, 540-545.

Matthijs, M.G., van Eck, J.H., Landman, W.J., Stegeman, J.A., 2003, Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. Avian Pathol. 32, 473-481.

McCullers, J.A., 2006, Insights into the Interaction between Influenza Virus and Pneumococcus. Clin. Microbiol. Rev. 19, 571-582.

Nakamura, K., Ueda, H., Tanimura, T., Noguchi, K., 1994, Effect of mixed live vaccine (Newcastle disease and infectious bronchitis) and Mycoplasma gallisepticum on the chicken respiratory tract and on Escherichia coli infection. J. Comp. Pathol. 111, 33-42.

Naqi, S., Thompson, G., Bauman, B., Mohammed, H., 2001, The exacerbating effect of infectious bronchitis virus infection on the infectious bursal disease virus-induced suppression of opsonization by Escherichia coil antibody in chickens. Avian Dis. 45, 52-60.

Navarini, A.A., Recher, M., Lang, K.S., Georgiev, P., Meury, S., Bergthaler, A., Flatz, L., Bille, J., Landmann, R., Odermatt, B., Hengartner, H., Zinkernagel, R.M., 2006, Increased susceptibility to bacterial superinfection as a consequence of innate antiviral responses. PNAS 103, 15535-15539.

O'Connell, R.M., Saha, S.K., Vaidya, S.A., Bruhn, K.W., Miranda, G.A., Zarnegar, B., Perry, A.K., Nguyen, B.O., Lane, T.F., Taniguchi, T., Miller, J.F., Cheng, G., 2004, Type I Interferon Production Enhances Susceptibility to Listeria monocytogenes Infection. J. Exp. Med. 200, 437-445.

Peighambari, S.M., Julian, R.J., Gyles, C.L., 2000, Experimental Escherichia coli respiratory infection in broilers. Avian Dis. 44, 759-769.

Philbin, V.J., Iqbal, M., Boyd, Y., Goodchild, M.J., Beal, R.K., Bumstead, N., Young, J., Smith, A.L., 2005, Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. Immunology. 114, 507-521.

Read, R.C., Goodwin, L., Parsons, M.A., Silcocks, P., Kaczmarski, E.B., Parker, A., Baldwin, T.J., 1999, Coinfection with Influenza B Virus Does Not Affect Association of Neisseria meningitidis with Human Nasopharyngeal Mucosa in Organ Culture. Infect. Immun. 67, 3082-3086.

Rothwell, L., Young, J.R., Zoorob, R., Whittaker, C.A., Hesketh, P., Archer, A., Smith, A.L., Kaiser, P., 2004, Cloning and Characterization of Chicken IL-10 and Its Role in the Immune Response to Eimeria maxima. J. Immunol. 173, 2675-2682.

Seki, M., Yanagihara, K., Higashiyama, Y., Fukuda, Y., Kaneko, Y., Ohno, H., Miyazaki, Y., Hirakata, Y., Tomono, K., Kadota, J., Tashiro, T., Kohno, S., 2004, Immunokinetics in severe pneumonia due to influenza virus and bacteria coinfection in mice. Eur. Respir. J. 24, 143-149.
Shtrichman, R., Samuel, C.E., 2001, The role of gamma interferon in antimicrobial immunity. Curr. Opin. Microbiol. 4, 251-259.
Slifka, M.K., Homann, D., Tishon, A., Pagarigan, R., Oldstone, M.B., 2003, Measles virus infection results in suppression of both innate and adaptive immune responses to secondary bacterial infection. J. Clin. Invest. 111, 805-810.
Speshock, J.L., Doyon-Reale, N., Rabah, R., Neely, M.N., Roberts, P.C., 2007, Filamentous Influenza A Virus Infection Predisposes Mice to Fatal Septicemia following Superinfection with Streptococcus pneumoniae Serotype 3. Infect. Immun. 75, 3102-3111.
Staeheli, P., Puehler, F., Schneider, K., Gobel, T.W., Kaspers, B., 2001, Cytokines of Birds: Conserved Functions -- A Largely Different Look. J. Interf. Cytok. Res. 21, 993-1010.
Storz, J., Lin, X., Purdy, C.W., Chouljenko, V.N., Kousoulas, K.G., Enright, F.M., Gilmore, W.C., Briggs, R.E., Loan, R.W., 2000, Coronavirus and Pasteurella Infections in Bovine Shipping Fever Pneumonia and Evans' Criteria for Causation. J. Clin. Microbiol. 38, 3291-3298.
Vandekerchove, D., Herdt, P.D., Laevens, H., Butaye, P., Meulemans, G., Pasmans, F., 2004, Significance of interactions between Escherichia coli and respiratory pathogens in layer hen flocks suffering from colibacillosis-associated mortality. Avian Pathol. 33, 298-302.
Wigley, P., Kaiser, P., 2003, Avian cytokines in health and disease. Rev. Bras. Cienc. Avic. 5, 1-14.
Wilson, R., Dowling, R.B., Jackson, A.D., 1996, The biology of bacterial colonization and invasion of the respiratory mucosa. Eur. Respir. J. 9, 1523-1530.
Withanage, G.S.K., Kaiser, P., Wigley, P., Powers, C., Mastroeni, P., Brooks, H., Barrow, P., Smith, A., Maskell, D., McConnell, I., 2004, Rapid Expression of Chemokines and Proinflammatory Cytokines in Newly Hatched Chickens Infected with Salmonella enterica Serovar Typhimurium. Infect. Immun. 72, 2152-2159.

1. Introduction

CHAPTER 7

Summarizing discussion

2. Novel tools to examine chicken immune responses
3. Vaccine components and route of administration
4. Modulation of the immune response
5. Concluding remarks

1. Introduction

Although vaccination has been one of the most powerful methods to protect people and animals against a wide range of infectious diseases for the past century, it is not clear what makes a vaccine a success or a failure. Many vaccines on the market these days provide a good recall immune response to challenge with the target pathogen, but fail to generate protective immunity to the pathogen for an extended period of time. Nowadays, new tools have become available to study the mechanisms involved in adequate induction of protective immunity. The general aim of this thesis was to gain insight into various aspects of the interaction between a vaccine, its target pathogen and the host immune response. A better knowledge of these interactions will allow for more effective vaccination strategies and vaccine designs.

The chicken, which is both the experimental and target species at the same time, was used to study these interactions. We identified three topics that in our view are instrumental in vaccine development and which formed the subjects of the chapters of this thesis. To recapitulate briefly, we first needed tools to monitor the immune response. Secondly, we required a better understanding of the context of the immune response. For this we had to keep the number of antigen-specific determinants involved in the experiments to a minimum. This was supposed to improve our possibilities to directly correlate the outcome of an immune response with the additional substances we provided to induce it, such as adjuvants. And finally, we wanted to gain insight into factors other than our own input variables that can modulate the immune response. In other words, we wanted to increase our understanding of how the nature of an immune response after vaccination can be different between two individuals, even if they both received the exact same vaccine and have the same genetic background.

2. Novel tools to examine chicken immune responses

In order to examine specific immune responses, we first had to develop tools that would allow us to interpret both the innate and the adaptive immune responses of our chosen host animal, the chicken, in more detail. In chapter 2 of this thesis, we successfully developed an ELISPOT assay and an intracellular cytokine staining (ICCS) assay for the detection of ChIFN-γ, which is a marker for T cell activity. The ELISPOT assay successfully detected T cell recall responses to Newcastle Disease Virus (NDV) in chickens vaccinated against this disease.

The ELISPOT assay was also tested using Infectious Bronchitis Virus (IBV) as the target pathogen, but we found unexpectedly early and high ChIFN-γ production after *in vitro* restimulation of chicken splenocytes with IBV, regardless of whether the chickens were immunized for IBV or not. To establish whether this early immune cell activation after incubation with IBV was an actual biologically valid effect or an assay artefact, we studied this phenomenon, as detailed in chapter 3. It was shown that the observed IBV-induced ChIFN-γ production was linked to the IBV itself and not to contaminating antigens in the virus preparation. The rapid IFN-γ production was species-specific and it was not an ELISPOT-related artefact, because similar observations were made using ELISA and quantitative RT-PCR (qPCR).

Type-I interferons, such as IFN-α and IFN-β, are closely connected to anti-viral activity, but it has been described that the type-II interferon IFN-γ is sometimes required in combination with type-I interferons for optimal anti-viral immune responses (Castilletti et al., 2005; Kumashiro et al., 2002). It is possible that this is

also the case for IBV.

An interesting alternative explanation would be that the IBV virus itself, or a viral product, acts as a superantigen. A similar phenomenon has already been reported for gammaherpesvirus and some other viruses, where a virally expressed secretion protein activates T cells without the need for professional antigen presentation (Evans et al., 2008). The rationale behind the production of a superantigen by the virus is not known, but it is tempting to speculate that the virus actively modulates the immune system to maintain persistent infection. In the case of the gammaherpesvirus, it is known that the superantigen M1 causes activation of a $CD8^+$ T cell subset that inhibits virus reactivation in immunocompetent hosts, thereby making sure that the viral genome remains latent and thus undetected by the host immune system. The possibility that IBV produces or is itself a viral superantigen would make this an interesting topic for future research.

3. Vaccine components and route of administration

In recent years it was shown that the route of administration can have a profound impact on the effectiveness of the immune response. In fact, the first contact between the pathogen and the innate immune system determines the eventual outcome of the specific immune response. This is because the innate immune system has so-called pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), complement receptors (CRs) and nucleotide-binding oligomerization domain (NOD) receptors, which recruit and activate dendritic cells (DCs) in a manner most suited for the type of infectious agent. The DCs in turn process the infectious agent and present it to lymphocytes, together with an array of co-stimulatory signals that provide additional instructions for the lymphocytes regarding which tissues to go to and the type of immune response that needs to be generated (Hoebe et al., 2004). As different sites in the body have different combinations of innate immune components, a vaccine can generate totally different immune responses when administered to different locations.

The impact of the administration route can be seen for instance in polio vaccines, where the intravenously administered polio vaccine generates antibodies only in the blood, but the orally administered version also generates antibodies in the intestinal tract, which is the primary entry and replication site of the virus. The oral vaccine therefore is far more effective in preventing person-to-person transmission. Unfortunately the oral vaccine virus is also more prone to revert to neurovirulence, which is why this version of the vaccine is generally not used in industrialized countries (MacLennan et al., 2004). Another example is influenza virus vaccination, where the intramuscularly or subcutaneously administered vaccine elicits a good serum antibody response but is poor in inducing respiratory mucosal antibodies and cell-mediated immunity, whereas the orally administered vaccine does prime both the humoral and cell-mediated immune components, with a much more long-lasting effect (Cox et al., 2004).

Apart from the route of administration, the format as well as the medium in which the antigen is offered to the immune system affects the outcome of the response (Leclerc, 2003). With regard to the vaccine format, it is clear that when we present an infectious agent to the immune system in its natural form, either attenuated or inactivated, the immune system will be confronted with parts of the agent that differ from those that are seen when we present pre-selected components that are normally shielded due to being inside the pathogen, actively

concealed by it, or produced after infection. This in turn can trigger different aspects of the innate and adaptive immune system, which results in different immune responses.

An example of the impact of such differences in presentation format can be seen in influenza vaccines. The currently approved influenza vaccines CIV (conventional inactivated virus) and LAV (live-attenuated virus) were compared with a vaccine consisting of the two primary viral surface proteins hemagglutinin (HA) and neuraminidase (NA). All three vaccines induced high anti-HA antibody titers, which protected well against the virus strain corresponding to the vaccine strain, but not to other HA subtypes. By contrast, the CIV and LAV failed to induce sufficient NA-antibody titers, possibly due to the HA protein being immunodominant, whereas the protein vaccine did induce high anti-NA titers (Brett and Johansson, 2005).

Another example is vaccination against whooping cough, caused by *Bordella pertussis* bacteria. Whole-cell pertussis vaccine (WCV) was compared with three vaccines consisting of selected *B. pertussis* virulence factors. Although all vaccines proved to be protective in mice, there were significant differences in elimination efficiency of bacteria from the trachea and lung (van den Berg et al., 2000).

With regard to the medium in which the antigens are presented to the immune system, selected antigens on their own are often poorly immunogenic, as are replication-deficient attenuated or killed whole pathogens. In such cases, the addition of adjuvants is required for the induction of an immune response. Traditionally these adjuvants are substances, unrelated to the selected target antigen, that help to elicit an immune response, which will then be directed against both the adjuvant component and the selected antigen component of the vaccine. These days it is generally accepted that micro-organisms also carry their own intrinsic adjuvant components, which are detected by the previously mentioned pattern recognition receptors (PRRs). Different adjuvants can modulate the immune response towards a different outcome, as was demonstrated when different TLR ligands were compared for their use as vaccine adjuvants (Weeratna et al., 2005). Besides adding separate immunogenic components to a vaccine, the selected antigens can also be incorporated into an immunogenic carrier, such as liposomes or microparticles (Scheerlinck and Greenwood, 2006), or in a bacterial or viral plasmid that encodes for the antigen of interest (van Drunen Littel-van den Hurk et al., 2004).

In theory, a proper choice of vaccine format and medium should produce a vaccine that will generate the desired immune response and protective immunity, regardless of the administration route. Our intention was to investigate the mechanisms behind the nature of the immune response induced by a vaccine. A key aspect of an effective vaccine, especially against respiratory pathogens, is the induction of a good humoral response, because antibodies form an important first defence against infection by a pathogen. However, many infectious agents have developed ways to avoid detection and neutralization by antibodies (Dorner and Radbruch, 2007). This is why we were especially interested in targeting cytotoxic T cell responses as well as the humoral response, because a cytotoxic T cell response is needed to expose intracellular pathogens that try to evade an effective immune clearance by hiding inside host cells (Stemberger et al., 2007).

For this, we required a system with limited complexity, to reduce the parameters that need to be taken into consideration when studying the immune response, and a vaccine component that is capable of eliciting a cytotoxic T cell response. The initial idea was to use peptides from the IBV spike and nucleocapsid

proteins that were selected for their fit into the MHC class I molecule of an inbred chicken line, White Leghorn line P2a ($B^{19/19}$). The choice to use selected peptides instead of the whole protein was made, because it is known from literature that administration of purified whole protein favours presentation via MHC class II molecules, resulting in a predominantly humoral immune response (Barrett and Rezvani, 2007; Machiels et al., 2002).

However, when a number of candidate peptides, selected for their MHC fit, were tested on their ability to induce an *in vitro* recall response in splenocytes of IBV-infected B^{19}/B^{19} chickens, an immune response to any of the peptides or pooled peptide batches was not detected. It is likely that the peptides alone are not sufficient to elicit a response, as mentioned previously. Adding an adjuvant could potentially fix this problem, but it might also introduce unpredictable effects to our study, such as non-specific bystander activation or modulation of the immune response in undesired ways.

We decided to use a novel technique of presenting antigens to the immune system, which is immunisation with a viral plasmid that contains the gene for the IBV nucleocapsid protein. The nucleocapsid protein was chosen because it is the protein that is the most conserved between different IBV strains, which hopefully means that the resulting plasmid will be effective against a range of IBV strains. The use of a plasmid to present this protein to the immune system allows the introduction of the protein into the appropriate processing machinery for MHC class I presentation and the resulting CMI response, and at the same time provides an immunogenic stimulus for induction of both humoral and CMI responses (DiCiommo and Bremner, 1998).

As shown in chapter 4, we did observe a protective effect of plasmid immunization to a subsequent IBV infection in some birds, based on clinical signs, and slightly elevated IBV-specific antibody responses in the immunized birds. Due to unexpectedly elevated ChIFN-γ levels after IBV infection in all the birds irrespective of their immunization status, which is a phenomenon that we already addressed in chapter 3, but at the time of these experiments it was not yet known to us, we were unfortunately not able to study IBV-specific responses.

It can be argued that a systemic immunization is not the optimal route to generate protective immunity to a respiratory pathogen like IBV, as the systemic levels of protein produced may not be high enough to induce local protection in the airways. The prime-boost regime that we applied in our study should hopefully compensate for this problem, because it increases the amount of protein and the antigen-specific response to it. Nevertheless, it would be interesting to compare systemic administration of the IBV plasmid with administration via the respiratory route, as this is the natural route of infection. The only consideration for this is that the orally or intranasally administered plasmid would need to be encapsulated somehow to avoid degradation, which could introduce unexpected modulation of the results due to adjuvant effects of the encapsulating material.

However, as discussed previously, with the right choice of antigens and context it should be possible to design a vaccine in such a way that it will trigger the desired immune response regardless of the site of administration. Studying the effects of the vaccine context will hopefully facilitate the design of such vaccines in the future.

4. Modulation of the immune response

Besides the components and the administration route of a vaccine, the immune status of the host at the time of administration can also play a big role in the vaccine efficacy. A special case is when the vaccine itself changes the immune parameters in such a way that other, normally quite harmless pathogens that are already present at the time of vaccine administration suddenly give rise to an opportunistic infection. One example of this is the poultry IBV vaccine strain H120, which was reported to facilitate and enhance experimental colibacillosis in broilers (Matthijs et al., 2003) to a similar extent as field IB viruses such as the virulent IBV strain M41 do.

When developing an effective vaccine it is therefore important to understand the factors that can cause such undesirable side effects, in order to prevent or limit their occurrence. In chapter 5 we examined if mechanical damage of the epithelium of the respiratory tract by the IBV vaccine virus could be a possible explanation for increased susceptibility to other pathogens. Although damage to the epithelium was indeed found in the trachea and the lungs due to the vaccine virus, this damage did not appear to predispose birds for a subsequent *E. coli* infection. Histological examination of respiratory tissues suggested that the underlying cause for the enhanced susceptibility was more likely to be found in alteration or modulation of innate immune responses.

In chapter 6 we looked more closely at the modulating effects of the vaccine on the host immune response, to see if these modulations impair the response to pathogens of a different nature than the intended target pathogen. We found that virulent IBV and the IBV vaccine virus did not impair the phagocytic ability or nitric oxide production of innate immune cells, but the IBV infection did cause a systemic change in cytokine expression patterns, which in all likelihood affected the kinetics of the immune response against other pathogens that were present during or shortly after vaccination.

These findings are an important lesson when designing a vaccine. Live attenuated IBV vaccines are widely used in the poultry industry (Cavanagh, 2005) and effectively protect against IBV field viruses, but this vaccination strategy can predispose chickens for secondary bacterial infections such as colibacillosis (Matthijs et al., 2003). This shows us that even when a vaccine is carefully designed and proven to provide optimal long-lasting protection against its target pathogen in a controlled experimental environment, as soon as the vaccine is applied in the field problems can arise due to the immune status of the host. Vaccine development therefore needs to take into account not only aspects of the infectious agent, such as target antigens and immunization route, but also the interplay between the chosen immunization strategy and the implications of the outcome of the immune response on the overall host immune status. Ideally a vaccine should provide both acute protection and induce long-lasting immune memory against the infectious agent, as well as prevent latent infection and animal-to-animal transmission, without preoccupying or altering the host immune defence to such a level that other pathogens can break through. Whether this is an achievable goal in practice remains to be seen.

5. Concluding remarks

The ELISPOT and intracellular cytokine staining assays for chicken IFN-γ allow a more detailed study of the adaptive immune response. As more cytokines in the chicken are cloned and antibodies are becoming available, these techniques

can be applied to a wider range of cytokines. It will also make it easier to study the effect of selected antigens of an infectious agent on the immune system, which will facilitate the search for good vaccine components.

The value of these techniques has already been demonstrated by unveiling a possible characteristic of IBV that to our knowledge has not been described before. This characteristic is the apparent ability to rapidly induce non-specific polyclonal T cell activation. The possibility that IBV might act as a superantigen is an intriguing topic for future study.

Using antigens of limited complexity is a prerequisite in immunological studies, if one wants to draw sensible conclusions about the context of the observed response. If the number of epitopes presented to the system becomes too large, it will not be possible to determine what the effect is of changes in the vaccine context, such as adjuvants, on the outcome of the immune response. For experimental purposes, we therefore need to have good control over the antigen-specificity in order to examine the vaccine context. Only when we have a good understanding of the context can we start combining different specific and non-specific components to create an appropriate vaccine.

Vaccines will usually require an adjuvant. Viral or bacterial plasmids are an interesting option in this respect, as they can present the antigen of interest to the immune system in a way that induces activation of both humoral and cytotoxic T cell responses. In poultry, DNA immunization is still in its infancy, partly due to the relatively high costs of such vaccination strategies compared to the use of live attenuated or inactivated pathogens. A potential big advantage of DNA-based vaccines over conventional vaccines in poultry is the possibility of vaccination in the egg, as DNA plasmids are not subject to neonatal tolerance or maternal antibodies (Haygreen et al., 2005).

When designing these vaccine strategies one should never lose sight of the fact that there are two major players involved in a successful vaccination. On the one hand there is the vaccine itself, the potential of which is determined by its ‘ingredients’ and the way in which it is administered. On the other hand there is the host immune system which, apart from having to properly process and respond to the vaccine, is also continuously exposed to other infectious agents that are often completely unrelated to the vaccine target. This means that a good vaccine should not only induce a long-lasting protective response to its target pathogen, but also make sure that it does not unfavourably modulate the host immune system to other pathogens in the process. Designing such vaccines will be a challenging and interesting field of study for many years to come.

References

Barrett, A.J., Rezvani, K., 2007, Translational mini-review series on vaccines: Peptide vaccines for myeloid leukaemias. Clin. Exp. Immunol. 148, 189-198.

Brett, I.C., Johansson, B.E., 2005, Immunization against influenza A virus: comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology 339, 273-280.

Castilletti, C., Bordi, L., Lalle, E., Rozera, G., Poccia, F., Agrati, C., Abbate, I., Capobianchi, M.R., 2005, Coordinate induction of IFN-alpha and -gamma by SARS-CoV also in the absence of virus replication. Virology 341, 163-169.

Cavanagh, D., 2005, Coronaviruses in poultry and other birds. Avian Pathol. 34, 439-448.
Cox, R.J., Brokstad, K.A., Ogra, P., 2004, Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand. J. Immunol. 59, 1-15.
DiCiommo, D.P., Bremner, R., 1998, Rapid, high level protein production using DNA-based Semliki Forest virus vectors. J. Biol. Chem. 273, 18060-18066.
Dorner, T., Radbruch, A., 2007, Antibodies and B cell memory in viral immunity. Immunity 27, 384-392.
Evans, A.G., Moser, J.M., Krug, L.T., Pozharskaya, V., Mora, A.L., Speck, S.H., 2008, A gammaherpesvirus-secreted activator of Vβ4+ CD8+ T cells regulates chronic infection and immunopathology. J. Exp. Med. 205, 669-684.
Haygreen, L., Davison, F., Kaiser, P., 2005, DNA vaccines for poultry: the jump from theory to practice. Exp. Rev. Vaccines 4, 51-62.
Hoebe, K., Janssen, E., Beutler, B., 2004, The interface between innate and adaptive immunity. Nat. Immunol. 5, 971-974.
Kumashiro, R., Ide, T., Sasaki, M., Murashima, S., Suzuki, H., Hino, T., Morita, Y., Miyajima, I., Ogata, K., Tanaka, E., Yoshida, H., Tanikawa, K., Sata, M., 2002, Interferon-[gamma] brings additive anti-viral environment when combined with interferon-[alpha] in patients with chronic hepatitis C. Hepatol. Res. 22, 20-26.
Leclerc, C., 2003, New approaches in vaccine development. Comp. Immunol. Microbiol. Infect. Dis. 26, 329-341.
Machiels, J.P., van Baren, N., Marchand, M., 2002, Peptide-based cancer vaccines. Sem. Oncol. 29, 494-502.
MacLennan, C., Dunn, G., Huissoon, A.P., Kumararatne, D.S., Martin, J., O'Leary, P., Thompson, R.A., Osman, H., Wood, P., Minor, P., Wood, D.J., Pillay, D., 2004, Failure to clear persistent vaccine-derived neurovirulent poliovirus infection in an immunodeficient man. Lancet 363, 1509-1513.
Matthijs, M.G., van Eck, J.H., Landman, W.J., Stegeman, J.A., 2003, Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. Avian Pathol. 32, 473-481.
Scheerlinck, J.P., Greenwood, D.L., 2006, Particulate delivery systems for animal vaccines. Methods 40, 118-124.
Stemberger, C., Neuenhahn, M., Buchholz, V.R., Busch, D.H., 2007, Origin of CD8+ effector and memory T cell subsets. Cell. Mol. Immunol. 4, 399-405.
van den Berg, B.M., David, S., Beekhuizen, H., Mooi, F.R., van Furth, R., 2000, Protection and humoral immune responses against Bordetella pertussis infection in mice immunized with acellular or cellular pertussis immunogens. Vaccine 19, 1118-1128.
van Drunen Littel-van den Hurk, S., Babiuk, S.L., Babiuk, L.A., 2004, Strategies for improved formulation and delivery of DNA vaccines to veterinary target species. Immunol. Rev. 199, 113-125.
Weeratna, R.D., Makinen, S.R., McCluskie, M.J., Davis, H.L., 2005, TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine 23, 5263-5270.

1. Inleiding

NEDERLANDSE SAMENVATTING

1. Inleiding

Vaccinatie heeft tot doel om langdurige immunologische bescherming op te wekken tegen infectieziekten. Dit wordt doorgaans bereikt door het aanbieden van een verzwakte of geinactiveerde versie van een ziekteverwekker, of geselecteerde componenten of produkten hiervan, aan het immuunsysteem. Nadat het immuunsysteem een succesvolle afweerreactie tegen deze vaccin-versie van de ziekteverwekker heeft gegenereerd, kan een langdurig “immuungeheugen” tegen de natuurlijke infectie aanwezig blijven. Dit immuungeheugen zorgt ervoor dat een nieuwe aanval van deze ziekteverwekker snel wordt ontdekt en afgestopt.

Hoewel vaccinatie al decennia lang erg succesvol gebleken is in de bestrijding van een groot aantal infectieziekten, zijn er nog altijd ziekten waartegen om onduidelijke redenen geen adequate of langdurige bescherming optreedt na vaccinatie. Het is daarom belangrijk meer kennis op te doen over de mechanismen die ten grondslag liggen aan het succes of het falen van een vaccin. Met de komst van nieuwe immunologische onderzoeksmethoden is ook onze kennis de laatste jaren op dit vlak gestaag gegroeid. Men is zich er nu van bewust dat een adequate immuunreactie na vaccinatie niet alleen het resultaat is van de juiste antigeen-specifieke vaccin-componenten, maar ook van de context waarin deze reactie opgewekt wordt. Het is daarom van belang dat bij vaccin-ontwikkeling niet alleen gekeken wordt naar produktie van antigeen-specifieke responsen, maar ook naar de invloed van de context waarin het vaccin wordt toegediend, zoals vaccin adjuvantia, toedieningsroute en de immuunstatus van de gastheer zelf.

Vaccinatie wordt niet alleen toegepast bij mensen, maar ook bij een grote verscheidenheid aan diersoorten, zoals huisdieren en productiedieren in de veehouderij. Voor vaccin-onderzoek is het daarom aantrekkelijk om te werken met een diersoort die geschikt is als onderzoeksmodel en tevens het doeldier vormt voor het te ontwikkelen vaccin. In dit proefschrift is gekozen voor de kip, omdat de pluimveesector economisch gezien wereldwijd een zeer belangrijke sector is, waar grote vraag bestaat naar vaccins die een goede bescherming bieden tegen een breed scala aan infectieziekten. Daarnaast is de kip traditioneel gezien een populair dier voor immunologisch onderzoek, waarbij de werkingsmechanismen van de specifieke afweer tegen infectieziekten grote gelijkenissen vertonen met die in de mens.

In het onderzoeksproject waarvan dit proefschrift het eindresultaat is, is geprobeerd om een beter inzicht te krijgen in de factoren die een rol spelen bij het wel of niet effectief zijn van een vaccin. In hoofdstuk 1 van dit proefschrift zijn drie aandachtspunten geïdentificeerd die in onze ogen bepalend zijn voor kennisverwerving en de ontwikkeling van een succesvol vaccin in de kip. Deze drie punten zijn A) de mogelijkheid tot gedetailleerde bestudering van antigeen-specifieke immuunresponsen; B) het gebruik van antigenen van geringe complexiteit om bestudering van de context van de immuunresponsen mogelijk te maken; en C) inzicht in het effect van de immuunstatus van de gastheer op de uitkomst van een vaccinatie. De daarop volgende hoofdstukken beschrijven de bevindingen die gedaan zijn met betrekking tot deze aandachtspunten.

2. Nieuwe technieken in pluimvee-onderzoek

Allereerst is het noodzakelijk om experimentele technieken te ontwikkelen waarmee lichaamsvreemde of antigeen-specifieke immuunresponsen in de kip gemeten en geanalyseerd kunnen worden, omdat de antigeen-specifieke immuunresponsen cruciaal zijn voor het verkrijgen van een langdurige bescherming

tegen infecties.

De technieken die hiervoor beschikbaar zijn bij pluimvee waren niet toereikend voor onze doeleinden. Daarom werden twee nieuwe technieken ontwikkeld voor de kip, de cytokine ELISPOT assay en de intracelulaire cytokine detectie assay (ICCS), voor de detectie van interferon-gamma (IFN-γ). IFN-γ is een eiwit dat een belangrijke rol heeft in ontstekingsreacties, en een toename in de produktie van dit eiwit is een algemeen geaccepteerde indicator voor een antigeen-specifieke immuunreactie. De ontwikkeling van deze nieuwe meetmethodes is beschreven in hoofdstuk 2.

Bij het ontwikkelen van deze technieken werd gebruik gemaakt van een infectiemodel met het respiratoire kippenvirus Infectious Bronchitis Virus (IBV). IBV is een virus bestaande uit slechts vier structurele eiwitten. Dit maakt IBV een aantrekkelijker doelwit voor antigeen-specifiek onderzoek dan structureel meer gecompliceerde virussen of bacteriën. Bovendien is IBV nauw verwant aan het humane SARS virus, waardoor inzicht in de werkingsmechanismen van dit virus mogelijk geëxtrapoleerd kan worden naar humane vaccin-ontwikkeling.

Tijdens deze infectiestudies werd een opmerkelijke waarneming gedaan. Het bleek dat blootstelling van kip immuuncellen aan IBV *in vitro* leidde tot een snelle en sterk verhoogde produktie van IFN-γ, zowel bij cellen van IBV-geïnfecteerde als bij ongeïnfecteerde kippen. Dit fenomeen wordt in meer detail beschreven in hoofdstuk 3. Hierbij bleek dat het waargenomen effect specifiek is voor IBV en het impliceert dat IBV mogelijk als een soort super-antigeen werkt. Eerder is al beschreven dat SARS een storm aan diverse signaal-eiwitten veroorzaakt. Het is niet onwaarschijnlijk dat IBV eenzelfde effect vertoont, en dit maakt IBV een interessant modelvirus voor SARS-studies.

3. Antigeen complexiteit

Met het beschikbaar hebben van technieken om antigeen-specifieke immuunresponsen te meten, is het belangrijk om alle experimenten uit te voeren onder goed gecontroleerde condities. Hiervoor zijn antigenen van geringe complexiteit noodzakelijk, want alleen met dergelijke antigenen kan een direct verband gelegd worden tussen de aangeboden antigeen-specifieke stimulans en de resulterende immuunrespons.

In eerste instantie werd gebruik gemaakt van geselecteerde eiwit-fragmenten (peptiden) van de ziekteverwekker, maar het bleek al snel dat deze peptiden totaal geen immuunreactie veroorzaakten. Waarschijnlijk beschouwt het immuunsysteem zulke peptiden niet als bedreigend en is een extra stimulans noodzakelijk om hen onder de aandacht van het immuunsysteem te brengen. Een dergelijke toegevoegde immuunstimulans, adjuvans genaamd, wordt vaak gebruikt in vaccins. Er zijn vele soorten adjuvantia, en hun effect op de immuunrespons kan erg verschillend zijn. Het is daarom belangrijk om een adjuvans te kiezen dat het vaccin in staat stelt om de meest effectieve immuunreactie op te wekken. Het toegevoegde effect van een adjuvans kan alleen bestudeerd worden als de immuunrespons tegen het antigeen-specifieke element van het vaccin onder controle is. Met andere woorden, als de antigeen-specifieke stimulans een geringe complexiteit heeft.

Bij vaccinatie tegen diverse infecties is het van belang dat er zowel een goede antilichaam productie als cytotoxische immuunreactie wordt gegenereerd. In hoofdstuk 4 worden de resultaten beschreven van een infectiestudie waarbij gebruik gemaakt is van een DNA plasmide dat dienst doet als adjuvans en tevens als

presentatieplatform voor het nucleocapside eiwit van IBV. Van DNA plasmides is bekend dat zij doorgaans zeer effectief zijn in het activeren van zowel de antilichaam productie als de cytotoxische component van het immuunsysteem. Immunisatie met het plasmide leidde tot een lichte toename in bescherming tegen IBV-infectie en verhoogde productie van IBV-antilichaam bij sommige kippen. Het gebruik van antigenen van geringe complexiteit stelt ons op deze wijze in staat om meer inzicht te krijgen in de effecten van toedieningsroute en adjuvans op de immuunreactie.

4. Modulatie van de immuunreactie

Naast de vaccin-componenten en de toedieningsroute van een vaccin, speelt de immuunstatus van de gastheer een belangrijke rol bij het uiteindelijke succes van vaccinatie. Zo kan bij een gastheer met een verzwakt immuunsysteem de vaccinatie minder effectief zijn doordat de benodigde immuunreactie tegen het vaccin niet optimaal tot stand komt en als gevolg daarvan een langdurig beschermende immuunstatus uitblijft. Aan de andere kant kan in een gezonde gastheer de vaccinatie zelf het immuunsysteem dusdanig beïnvloeden, dat ziekteverwekkers die normaliter geen probleem opleveren opeens aanleiding kunnen geven tot opportunistische infecties. Een voorbeeld hiervan is het IBV pluimvee-vaccin H120, dat net als de IBV veldvirus stam M41 een verhoogde ontvankelijkheid voor experimentele infectie met *Escherichia coli* kan veroorzaken bij kippen. Het is daarom belangrijk om meer inzicht te krijgen in de factoren die ten grondslag liggen aan zulke onwenselijke bijverschijnselen van vaccinatie.

In hoofdstuk 5 en 6 van dit proefschrift is gekeken naar de bijdrage van verschillende factoren die een rol kunnen spelen in deze toegenomen ontvankelijkheid voor *E. coli* na IBV infectie of vaccinatie. In hoofdstuk 5 wordt onderzocht of door IBV veroorzaakte mechanische schade aan de ademhalingswegen een oorzaak is van infectie met *E. coli* bacteriën. Hoewel inderdaad schade kon worden waargenomen aan de luchtwegen na IBV infectie en vaccinatie, bleek dit geen waarneembare invloed te hebben op de mate van *E. coli* infectie. Uit nadere bestudering van weefselmonsters van de luchtwegen ontstond de suggestie dat de onderliggende reden voor de toegenomen ontvankelijkheid mogelijk lag in ontregeling van innate immuunresponsen.

In hoofdstuk 6 is onderzocht of blootstelling aan IBV invloed heeft op bepaalde aspecten van het immuunsysteem. Uit deze studies kwam naar voren dat belangrijke afweermechanismen van macrofagen, zoals fagocytose en NO produktie, niet werden aangetast door het virus. Wel zorgde infectie of vaccinatie met IBV voor een systemische verandering in de produktie van bepaalde cytokines, wat naar alle waarschijnlijkheid de weerstand tegen *E. coli* infectie aantast.

Hieruit blijkt dat bij vaccin-ontwikkeling niet alleen gekeken moet worden naar de te bestrijden ziekteverwekker, maar ook naar het effect van het vaccin op de algehele immuunstatus van de gastheer.

5. Conclusies

De in dit proefschrift beschreven nieuwe technieken voor het analyseren van antigeen-specifieke immuunreacties in de kip kunnen het inzicht in de interacties tussen pathogeen en gastheer vergroten. Dit heeft inmiddels al geresulteerd in de ontdekking dat IBV een snelle niet-specifieke immuun activatie veroorzaakt, een

eigenschap die mogelijk implicaties heeft voor onderzoek naar zowel IBV als het aanverwante humane SARS virus. Bovendien kunnen ze een nuttige bijdrage leveren aan vaccin-ontwikkeling voor pluimvee, doordat het nu mogelijk is om verschillende vaccinatie-strategieën te onderzoeken in een opzet waarbij een goede controle bestaat over de antigeen-specifieke component. DNA immunisatie biedt een interessant alternatief voor gangbare vaccinatie-strategieën bij pluimvee. Het induceert zowel cytotoxische als antilichaam responsen, en doet gelijktijdig dienst als adjuvans en als vervoermiddel voor de pathogeen-specifieke component waartegen de afweerreactie moet plaatsvinden.

Bij de ontwikkeling van nieuwe vaccinatie-strategieën is het van belang dat de immuunstatus van de gastheer niet uit het oog verloren wordt. Vaccinatie kan een modulerende werking uitoefenen op het immuunsysteem van de gastheer, wat in sommige gevallen kan leiden tot een verhoogde ontvankelijkheid voor opportunistische infecties. Alleen als de beoogde vaccinatie-strategie en de immuunreactie van de gastheer goed op elkaar zijn afgestemd, kan vaccinatie de balans in het voordeel van de gastheer doen doorslaan.

Dankwoord

Juist als je denkt dat het werk er bijna op zit, dringt het besef door dat het belangrijkste gedeelte van het proefschrift nog geschreven moet worden: het dankwoord. Zonder twijfel de meest gelezen en aan zorgvuldige dissectie onderworpen passage van dit gehele boekje. Het lijkt mij dan ook verstandig om bij deze alvast iedereen die ik persoonlijk een exemplaar in de hand gedrukt heb of dit via een andere weg heb doen toekomen te bedanken voor de bijdrage aan dit proefschrift of aan mijn geestelijke gezondheid gedurende de afgelopen jaren. Mocht je onverhoopt je naam niet terugvinden in dit ongetwijfeld veel te korte overzicht, dan is dit niet omdat ik deze bijdragen niet op waarde heb geschat, maar simpelweg omdat ik een geheugen als een zeef heb.

Op een prominente eerste plaats wil ik Lonneke Vervelde bedanken. Lonneke, ik durf met een gerust hart te stellen dat zonder jouw steun en inzet dit boekje hoogstwaarschijnlijk nooit bestaan zou hebben. Je nam een op zeker moment lichtelijk zwalkende AIO stevig bij de oren en hebt hem met veel geduld en toewijding naar de eindstreep gesleurd. Door jouw enthousiasme en aanmoedigingen heb ik het geloof erin gehouden en ik hoop dat dit boekje een Killer Queen-waardig resultaat van die inspanningen is. Ik weet dat je gemiep niet op prijs stelt, dus ik zal het hier verder afdoen met een simpel “dank je”. Schrijnend ontoereikend als het is, je weet waar het vandaan komt.

Daarnaast wil ik ook Evert Hensen bedanken. Evert, het was jouw visie en bijna encyclopedische kennis van de immunologie die de basis voor dit project vormden. Je gaf me het vertrouwen om jouw ideeën en kennis te boetseren tot een volwassen onderzoeksproject. Ik weet niet of ik je vertrouwen te allen tijde recht gedaan heb, maar ik hoop dat je in dit proefschrift genoeg van jouw visie terug vindt om dat vertrouwen terug te betalen. Het is een interessante reis geweest en, al zijn we de *Salmonella* onderweg lichtelijk uit het oog verloren en hebben we een flinke tijd met vogelgriep op bed gelegen, de eindbestemming is hopelijk de moeite waard gebleken.

Willem van Eden, mijn promotor, hoewel het onderwerp van mijn proefschrift uiteraard niet direct in je aandachtsveld ligt en ik, ondanks dappere pogingen van jouw kant, er nooit echt in geslaagd ben Hsp moleculen als een volwaardig thema in de kippenwereld onder de aandacht te brengen, heb ik je geloof in mijn kunnen altijd gewaardeerd. Ook toen door interne en externe omstandigheden vertragingen de kop op staken heb je me steevast de kans gegeven om dit project te voltooien, waarvoor dank.

Robbert van der Most, helaas was de aantrekkingskracht van het buitenlandse avontuur te groot om je voor de vakgroep te behouden, maar bedankt voor al je begeleiding in mijn vroege AIO-jaren en je inspirerende ideeën en inventieve oplossingen.

Ook vele malen dank aan de kippengroep. Toen ik als AIO begon waren de enige kippensporen op de afdeling de stukjes kipfilet op de pizzas tijdens de journal clubs, toen ik wegging was er zowaar een volwaardige pluimvee onderzoeksgroep. Daphne en Sylvia, dank je voor al jullie hulp, in het lab en soms natuurlijk ook als bliksemafleider bij projectgroep-gesprekken. Naast al de praktische hulp bij het vele bacteriën uitplaten, trachea-ringen cultiveren en eitjes pellen was het ook een stuk gezelliger werken! Peter, helaas raakte je pas echt bij mijn werk betrokken tegen het einde van mijn AIO-termijn en heb ik voornamelijk via e-mails met je gecommuniceerd, maar het werk dat je verzet hebt voor sommige van onze papers is indrukwekkend. Cheers mate!

En uiteraard de andere Peter, Peter van Kooten. Je was een onschatbare bron

van ervaring en suggesties in het lab. En daarnaast natuurlijk ook altijd in voor een feestje! Het was fijn om met je te werken, en ik spiegel mij in mijn huidige baan graag aan jouw lichtend voorbeeld.

Mijn kamergenoot Edwin, je stond altijd voor me klaar als ik ergens hulp bij nodig had of gewoon voor een onderhoudend gesprek. Ook dank aan mijn andere kamergenoten voor een zeer gezellige tijd, met name: Ad, (bijna) eenzame strijder tegen de oprukkende niet-veterinaire hordes in het diergeneeskundig onderzoek; Ildiko, voor gezelligheid en geweldige kookkunsten; en Marieke, voor nog meer gezelligheid.

Verder dank aan alle mede-AIO's in de afdeling door de jaren heen: Teun (later begonnen, eerder klaar, het begon bijna een trend te worden! Dank je voor je hulp en advies vanuit de vuurlinie), Aad (Aadjeuhhhhh!!!! Ga ik je alsnog verslaan op de finishlijn, ouwe reus? Het lijkt Feyenoord wel), Lotte, Erwin, Esther, Suzanne, Ernst, Merel, Elmieke, en nu vergeet ik er ongetwijfeld nog wel een aantal (sorry!).

Dank uiteraard ook aan mijn andere collega's in de vakgroep Immunologie die me met raad en daad terzijde gestaan hebben, ondermeer maar niet uitsluitend: Ger (rock on!), Victor, Ruurd, Femke, Josée, Corlinda, Mayken, Mieke, Daphne (van Oostenrijk), Danielle, Marije, en Marca.

De Engelsman John Donne zei ooit: "no man is an island" (behalve natuurlijk, zoals een Engelse komiek ooit opmerkte, de Isle of Man), en hetzelfde geldt uiteraard voor onderzoeksgroepen. Gedurende mijn promotie-onderzoek heb ik met veel plezier samengewerkt met mensen van verschillende departementen binnen en buiten de faculteit. Met name de afdeling Pluimveegezondheidszorg is voor mij een cruciale bron van informatie, hulp en onderzoeksmateriaal geweest.

Allereerst moet ik dan natuurlijk Marius Dwars bedanken, met wie ik al vroeg tijdens mijn project in contact kwam en die mij vooral in de begin-fase, toen ik letterlijk van de kip noch het ei wist, wegwijs heeft gemaakt in de wondere wereld van het pluimvee. Lange tijd was jij mijn voornaamste milt- en bloed-dealer, en menige ochtend heb ik met je door kippenhokken gesjouwd en mogen genieten van je geweldige uiteenzettingen over kippen, het leven, het universum en alles. Het was mij een waar genoegen.

Mieke, bedankt voor de zeer produktieve samenwerking in de laatste maanden van ons AIO-schap. Het was voor ons allebei een intensieve eindspurt, maar het was de moeite dubbel en dwars waard. Gedeelde stress is halve stress!

Jo, zoals jou maken ze ze niet meer. Een enorme en langdurig gecultiveerde kennis van pluimvee, en nooit te beroerd om zelf je handen vuil te maken. Wanneer jij met je pen los ging op de papers van Mieke en mij was je een onvermurwbare criticus, maar je had het wel altijd bij het rechte eind. Ook heb je belangrijke deuren voor me geopend in de faculteit, waardoor ik mijn experimenten veel produktiever en efficiënter kon uitvoeren. Ook bedankt aan Arjan en Annemarie voor waardevolle discussie en hulp. En natuurlijk aan alle dierenverzorgers die zorg hebben gedragen voor mijn kippen en geholpen hebben met het uitbroeden van eieren, met name Freek en Patricia.

Daarnaast wil ik ook alle (ex-)leden van de borrelcommissie hartelijk danken voor de mooie tijden: Kirezi, Margreet, Lieke, Tom, Frans, Teun (alweer) en andere betrokkenen, evenals alle collega's van andere vakgroepen die me door de jaren heen geholpen hebben of me de kroeg in hebben gesleept.

Vrienden en familie, jullie hebben me door de jaren heen altijd gesteund en voor de soms broodnodige afleiding gezorgd. Dank met name aan David en aan de

Mannuh (vrij naar Arnoud): Lars, Arnoud, Chris, Jasper, Sjoerd, Merijn, Robert-Jan, Roel en Floris, en natuurlijk ook aan Matthijs en Igor, voor zuupe, slap geklets, voetbal, muziek, films, en algehele persoonlijke ontwikkeling.

Mijn naaste familie: pa en ma, mijn zus Yvonne, John en de neefjes (en mijn nieuwe nichtje!), bedankt voor jullie onvoorwaardelijke steun, interesse en betrokkenheid.

En tenslotte mijn lieve Vikki. Het is de afgelopen jaren niet altijd even gemakkelijk geweest voor ons, eerst vanwege de afstand en later vanwege de vele uren en dagen die ik in dit proefschrift heb gestoken, maar jij bent altijd in mij blijven geloven en hebt mij onverminderd gesteund in hoogte- en dieptepunten. Dank je voor alles en nog meer. Het zit er nu echt op.

“I may not have gone where I intended to go,

but I think I have ended up where I needed to be.”

~ Douglas Adams ~

Curriculum vitae

Markus Petrus Ariaans werd geboren op 5 mei 1977 in Nijmegen. In 1995 behaalde hij het VWO diploma aan het Canisius College Mater Dei in Nijmegen. In datzelfde jaar begon hij met de studie Fundamentele Biomedische Wetenschappen aan de Universiteit Utrecht. Deze studie rondde hij met succes af in 2000, met afstudeerstages bij de vakgroep Vergelijkende Fysiologie & Neuroethologie (prof. W.A. van de Grind) en Immunologie (prof. T. Logtenberg). In 2001 startte hij zijn promotieonderzoek aan de Universiteit Utrecht, waarvan de resultaten beschreven zijn in dit proefschrift. Dit onderzoek werd uitgevoerd op de afdeling Immunologie, departement Infectieziekten en Immunologie van de faculteit Diergeneeskunde, onder begeleiding van dr. Evert Hensen en dr. Lonneke Vervelde, met professor dr. Willem van Eden als promotor. Vanaf april 2006 is hij werkzaam als laborant bij de Cardiovascular Research Unit in de School of Medicine & Biomedical Sciences van de University of Sheffield, Groot-Brittannië.

List of publications

Mark P. Ariaans, Ines Pereira, Robbert G. Van der Most, Evert J. Hensen, Lonneke Vervelde. DNA immunization against Infectious Bronchitis Virus nucleocapsid. *Manuscript in preparation.*

Mark P. Ariaans, Peter M. van de Haar, Evert J. Hensen, Lonneke Vervelde. Infectious Bronchitis Virus induces acute interferon-gamma production through polyclonal timulation of chicken leukocytes. *Submitted.*

Mark P. Ariaans, Peter M. van de Haar, John W. Lowenthal, Willem van Eden, Evert J. Hensen, Lonneke Vervelde. ELISPOT and intracellular cytokine staining: novel assays for quantifying T cell responses in the chicken. Dev. Comp. Immunol. 2008; 32(11): 1398-1404.

Mark P. Ariaans, Mieke G.R. Matthijs, Daphne van Haarlem, Peter M. van de Haar, Jo H.H. van Eck, Evert J. Hensen, Lonneke Vervelde. The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after Infectious Bronchitis Virus infection. Vet. Immunol. Immunopathol. 2008; 123(3-4): 240-250.

Mieke G.R. Matthijs, Mark P. Ariaans, R. Marius Dwars, Jo H.H. van Eck, Annemarie Bouma, Arjan Stegeman, Lonneke Vervelde. Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with *E. coli*. *Accepted for publication in Vet. Immunol. Immunopathol.*

Antonio Scibelli, Robbert G. van der Most, Johan A. Turkstra, Mark P. Ariaans, Ger Arkesteijn, Evert J. Hensen, Rob H. Meloen. Fast track selection of immunogens for novel vaccines through visualisation of the early onset of the B-cell response. Vaccine 2005; 23(16): 1900-1909.